FACULTY OF VETERINARY MEDICINE
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INNOVATIONS IN RAPID *MYCOPLASMA BOVIS* DIAGNOSTICS WITH MALDI-TOF MS AND NANOPORE SEQUENCING

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“Perfection is achieved, not when there is nothing more to add,

but when there is nothing left to take away”

Antoine de Saint-Exupéry

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LIST OF ABBREVIATIONS

ADM	Agar dilution method
AID	Anti-inflammatory drugs
AFLP	Amplification fragment length polymorphism
AMR	Antimicrobial resistance
AMU	Antimicrobial use
AP-PCR	Arbitrarily primed PCR
AST	Antimicrobial susceptibility testing
BAL	Bronchoalveolar lavages
BALf	Bronchoalveolar lavage fluid
BLCM	Bayesian latent class model
BMD	Broth microdilution
BRD	Bovine respiratory disease
BTM	Bulk tank milk
BVDv	Bovine viral diarrhea virus
CBP	Clinical breakpoint
CCU	Colour changing units
CFU	Colony forming units
cgMLST	Core genome MLST
cgSNP	Core genome single nucleotide variant
CLSI	Clinical and Laboratory Standards Institute
COVID-19	SARS-CoV-2
DGGE	Denaturing gradient gel electrophoresis
DNS	Deep nasal swab
DOXY	Doxycycline
ECOFF	Epidemiological cut-off
ELISA	Enzyme-linked immunosorbent assays
ENRO	Enrofloxacin
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FLOR	Florfenicol
GAM	Gamithromycine
GC	Guanine-cytosine
GEN	Gentamicin

GWAS	Genome-wide association study
HRM	High resolution melting curve analysis
ICEs	Integrative conjugative elements
IHL	In-house library
IM	Intramuscularly
IS	Insertion sequence
ISM	Iterative statistical method
LR	Likelihood ratio
MALDI-TOF MS	Matrix-assisted laser desorption-ionization – time of flight mass spectrometry
MBT-ASTRA	MALDI Biotyper-Antibiotic Susceptibility Test Rapid Assay
Melt-MAMA	Melt curve analysis of mismatch amplification mutation assays
MIC	Minimum inhibitory concentration
MICEs	*Mycoplasma* integrative conjugative elements
MLST	Multi-locus sequence typing
MLVA	Multiple-locus variable-number tandem repeat
MPC	Mutant prevention concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSP	Main spectra profiles
MSW	Mutant selection window
NGS	Next-generation sequencing
NRI	Normalized resistance interpretation
NSAID	Non-steroidal anti-inflammatory drugs
nWT	Non-wild type
OIE	World Organization for Animal Health
ONT	Oxford Nanopore Technologies/nanopore sequencing
OXY	Oxytetracycline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PPLO	Pleuropneumonia-like organisms
PURE-LAMP	Procedure for Ultra Rapid Extraction and Loop-mediated isothermal Amplification
QRDR	Quinolone resistance-determining region

RAPD	Random amplification of polymorphic DNA
RIMM	Rapid identification of *Mycoplasma bovis* with MALDI-TOF MS
SC	Subcutaneously
Se	Sensitivity
SMRT	Single Molecule Real-Time
SNP	Single Nucleotide Polymorphism
Sp	Specificity
ST	Sequence type
TIA	Tiamulin
TIL	Tilmicosine
TTA	Transtracheal aspiration
TTW	Transtracheal washe
TYL	Tylosine
UCC	Unit changing color
Vsps	Variable surface lipoproteins
wgMLST	Whole genome MLST
WGS	Whole genome sequencing
wgSNP	Whole genome single nucleotide variant
WHO	World Health Organization
WT	Wild type

PREFACE

The recent viral pandemic in humans has made it clear to millions of people how crucial rapid diagnostics and applied biosecurity are in infectious disease control. Worldwide, cattle farmers and their veterinarians almost continuously face a similar challenge in their animals related to different and often simultaneous respiratory tract infections. Among these infections, *Mycoplasma bovis* is absolute priority. *M. bovis* is highly contagious and a leading indication for antimicrobial mass medication. Treatment is difficult as they are inherently resistant against many antimicrobials and can evade the immune response of the body facilitating chronic, persistent infections.

Today, *M. bovis* control is still problematic. Effective biosecurity is hampered by incomplete epidemiological knowledge on *M. bovis*, especially concerning the existence of different strains and their spread between the dairy, beef and veal sector. Currently, using routine culturing, it takes more than a week to get a test result for *M. bovis* and susceptibility testing is not available. Alternative techniques like qPCR and antibody ELISA are often too expensive, lack sensitivity or show interpretative difficulties. Both for therapeutic outbreak management and more effective biosecurity, having rapid, accurate, and reasonably prized diagnostic tests available is crucial.

Thriving on the technological evolution in the field of diagnostics, this doctoral thesis explores the possibilities of MALDI-TOF MS and nanopore sequencing to make *M. bovis* diagnosis more fast and reliable. Epidemiological results obtained by these new protocols were also incorporated, since prevention of spread is at least as essential as treatment to combat this resourceful pathogen.

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION ON *MYCOPLASMA BOVIS*

Characteristics of *Mycoplasma* species

Mycoplasma bovis, previously known as *Mycoplasma agalactiae var. bovis* or *M. bovimastitidis* (Askaa and Erno, 1976), is one of the many known *Mollicutes* species (Razin, 1978). The class of the Mollicutes received its name due to the loss of their cell wall during evolution (molli: soft; cutis: skin) (Razin and Hayflick, 2010). More than 190 *Mycoplasma* species are currently documented (Rottem, 2003). These prokaryotes are among the smallest self-replicating organisms in the world (200-300 nm in diameter) (Razin, 1996), containing a genome length of 0.6-1 Mbp, with a low guanine-cytosine (GC) content of approximately 25-30% (Thompson et al., 2011; Kumar et al., 2020). The genome length is very small, in comparison to other bacteria, such as *Escherichia coli*, of which the bacterial genome size is 4.6 Mbp (Travisano, 2001). The limited genomic information of *Mycoplasma* species restricts its metabolic activities, resulting in the requirement of highly species-specific environments for growth. The necessary supplements, like amino acids, lipids, and energy sources such as pyruvate, arginine or urea, are usually withdrawn from the host (Rosenbusch, 1994; Masukagami et al., 2017). However, even under optimal circumstances, the growth rate of *Mycoplasma* species *in vitro* is very low (Miles, 1992; Rosenbusch, 1994; Razin and Hayflick, 2010).

Mycoplasma species are mostly host specific, although they are occasionally able to colonize other hosts (Rottem, 2003). *M. bovis* is mostly a pathogen of cattle, causing many different clinical diseases, such as bovine respiratory disease (BRD), arthritis and otitis in calves (Maunsell and Donovan, 2009) and mastitis and arthritis in adult cattle (Maunsell et al., 2011). However, next to affecting cattle, *M. bovis* has also been responsible for clinical signs in other ruminants, such as bisons (Janardhan et al., 2010), white-tailed deer (Dyer et al., 2004), mule deer (Register et al., 2019), pronghorn (Malmberg et al., 2020), sheep (Kumar et al., 2012), and goats (Rodríguez et al., 2000). Other possible carriers are rabbits (Boucher et al., 2001), poultry (Ongor et al., 2008), and in exceptional cases possibly even humans (Madoff et al., 1979). Many other *Mycoplasma* species can be isolated from cattle, such as the extremely pathogenic *Mycoplasma mycoides* subsp. *mycoide*s causing contagious bovine pleuropneumonia (Nicholas and Ayling, 2008). This pathogen is eradicated in Europe, but still a huge problem in Africa (Dudek et al., 2021). For several other *Mycoplasma* species (*e.g. M. bovirhinis*, *M. dispar*, *M. canis*, ..) the degree of pathogenity in cattle is not always clear.

Economic impact of *M. bovis* infections in cattle

The economic impact of *M. bovis* infections in cattle is very difficult to determine and only few studies have tried to estimate the cost. Before the 21st century, production losses due to *M. bovis* diseases were already counting for 32 and 108 million dollar per year in the American beef and dairy industry, respectively (Rosengarten and Citti, 1999). Production losses that are associated with *M. bovis* are for example a reduction in carcass quality, average milk production, and average daily gain (Rosengarten and Citti, 1999; Pardon et al., 2013; Francoz et al., 2015). The additional costs of losses to antimicrobial therapy, extra labor, mortality, culling, diagnostic tests and control measures were not included in this study. Another cost that can be included and is mostly forgotten, is the additional housing and feedings costs, due to lower average daily gain and extra time required to reach expected slaughter weight, due to the chronic nature of *M. bovis* infections (Maunsell et al., 2011; Calcutt et al., 2018). In Europe, it was estimated that 576 million euros per year were lost due to BRD of which at least 25% was attributable to *M. bovis* (Nicholas et al., 2000). These numbers are already quite outdated, but new estimates have not been published recently. Nevertheless, since the prevalence of *M. bovis* has probably increased the last few decades, and taking inflation into account, current costs due to *M. bovis* infections are probably greatly surpassing earlier estimations.

Prevalence

After the first isolation of *M. bovis* in the United States in 1961 from milk (Hale et al., 1962), this pathogen was rapidly observed in Europe and other continents as well (Nicholas et al., 2008) with currently a higher prevalence in central and Southern Europe compared to the Scandinavian countries (Gille, 2018). Finland was the last European country to get infected in 2012 by purchase of imported cattle (Haapala et al., 2018). Next to Finland, New-Zealand was able to stay *M. bovis* free for a very long time, but also got infected in 2017 by a currently unresolved infection route. There seems to be an increased prevalence of *M. bovis* in the last years (Kusiluka, 2000; Passchyn et al., 2012; Gille et al., 2018a). However, at world scale it is difficult to interpret and compare the role of *M. bovis*, because of the variety of samples and diagnostic methods used in prevalence studies. Very diverse prevalence numbers have been reported in Europe (0-100%), depending on the diagnostic methods and study population used (Gille, 2018). In Belgium for example, over the last few years the prevalence of *M. bovis* was 32% in dairy cattle by combined diagnostics of antibody enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR) on bulk tank milk (Gille et al., 2018a),

while a previous study showed only 1.5% prevalence by culture on bulk tank milk (Passchyn et al., 2012). Another Belgian study showed 33% of the herds to be positive by PCR on bronchoalveolar lavages (BAL) obtained in acute respiratory outbreaks on conventional beef and dairy farms (Pardon et al., 2020). In contrast, 100% of the veal herds endure exposure to *M. bovis* as reflected in their specific *M. bovis* antibody titers (Pardon et al., 2011). These prevalence numbers in the veal sector are in line with studies in other countries, such as Italy and France (Arcangioli et al., 2008; Radaelli et al., 2008) and nurture the fear that the veal sector, currently combining high antimicrobial use and facing high *M. bovis* prevalence, are a reservoir for multi-resistant *M. bovis* isolates (Jarrige et al., 2017; Bokma et al., 2019a).

Pathogenesis

Various virulence factors of *M. bovis* have been described (reviewed by Perez-Casal, 2020), but the most important ones are probably the variable surface lipoproteins (Vsps). These lipoproteins are used to adhere to mucosal surfaces (Lysnyansky et al., 1999; Sachse et al., 2000). *M. bovis* is mostly adhering to the mucosal surfaces of the upper respiratory tract (nasal cavity until tracheal bifurcation) and colonizing the tonsils (Miles, 1992; Maunsell and Donovan, 2009; Maunsell et al., 2011). However, it can also colonize the mucosal surfaces of the urogenital tract and the conjunctiva (Pfützner and Sachse, 1996; Levisohn et al., 2004). Another commonly colonized organ is the udder (Bennett and Jasper, 1980; Pfützner and Sachse, 1996). After colonization, multiplications at the site of infection will take place, and hematological spread of the pathogen to other organs is possible (Figure 1) (Bennett and Jasper, 1980; Maunsell and Donovan, 2009). Shedding of *M. bovis* mostly occurs from the upper respiratory tract and the udder (Bennett and Jasper, 1980; Soehnlen et al., 2012), but can for example also occur in semen (Haapala et al., 2018).

Once *M. bovis* adheres to the mucosal surfaces, an immune response is initiated (Rottem, 2003). However, *M. bovis* can evade both the innate and adaptive response (Kauf et al., 2007; Buchenau et al., 2010; Jimbo et al., 2017). Expression of the Vsps can be changed spontaneously by *M. bovis* and may allow to avoid the immune response (Lysnyansky et al., 1999; Buchenau et al., 2010). Next to this, *M. bovis* is able to persist intracellularly (*e.g.* lymphocytes, embryonic turbinate cells, and erythrocytes) (Van Der Merwe et al., 2010; Bürki et al., 2015; Nunoya et al., 2020).

Figure 1. Adherence and colonization sites of *M. bovis* marked by an asterisk: nares, eyes, brain, bulla tympanica, pharyngeal tonsillae, lung, heart, joints, abdomen, udder and urogenital tract. Also, hematological spread is possible (not shown).

Intracellularly, *M. bovis* is able to evade the humoral immune response and antimicrobial agents that are not able to penetrate host cells (Razin and Hayflick, 2010). Next to this, *M. bovis* is also able to evade the cellular immunity by impairing the viability of neutrophils, lymphocytes and macrophages (Vanden Bush and Rosenbusch, 2002; Suleman et al., 2016; Jimbo et al., 2017; Zhao et al., 2021). Variation in neutrophil phagocytizing and killing of *M. bovis* by different strains of *M. bovis* has been described (Wiggins et al., 2011; Alabdullah et al., 2015, 2018). Finally, *M. bovis* is also able to produce biofilms depending on the expression of Vsps, making it more resistant against environmental stress (McAuliffe et al., 2006). No influence of biofilm formation on the susceptibility of *M. bovis* to some antimicrobials was observed in the study of McAuliffe et al. (2006). Although in biofilm producing *Mycoplasma hyopneumoniae* isolates, an increase in minimum inhibitory concentration (MIC) values against different antimicrobials was observed (Tassew et al., 2017). The capacity of circumventing the immune response together with the ability to produce biofilms, can contribute to the chronic and persistent nature of *M. bovis* infections (Buchenau et al. 2010; Razin and Hayflick, 2010).

M. bovis associated diseases

Besides *M. bovis*, other *Mycoplasma* species (*e.g. arginini, M. alkalescens, M. canadense, M. californicum, M. canis, M. bovirhinis, M. bovigenitalium* and *M. dispar*) can be isolated from cattle (Thomas et al., 2002; Gioia et al., 2021). However, literature is mostly conflicting about the pathogenicity of these mycoplasmas. Therefore it is very important to identify and distinguish *M. bovis* from other less or nonpathogenic *Mycoplasma* species. In addition, mixed infections with different *Mycoplasma* species or with other bacterial (*e.g. Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* or viral (*e.g.* bovine viral diarrhea virus (BVDv), bovine respiratory syncytial virus, bovine parainfluenza type 3) pathogens, are frequently reported (Thomas et al., 2002; Szacawa et al., 2015; Animal and Plant Health Agency, 2016; Pardon et al., 2020). Nevertheless, the main focus in this thesis will be on *M. bovis* as it is widely accepted as a primary pathogen, causing pneumonia, arthritis, otitis, mastitis, and other less occurring manifestations of disease (Maunsell and Donovan, 2009; Haapala et al., 2019; Oliveira et al., 2020), which will be shortly illustrated in the next subchapters.

Pneumonia

Pneumonia by *M. bovis* is seen in all cattle sectors and all ages of animals (Maunsell and Donovan, 2009; Maunsell et al., 2011). Nevertheless, the contribution of *M. bovis* to acute and chronic BRD is higher in production systems relying on animal purchase like the veal calf or feedlot industry (Boothby et al., 1983; Caswell et al., 2010; Pardon et al., 2011). *M. bovis* is also involved in BRD outbreaks in dairy and beef calves in winter time (Pardon et al., 2020). Clinical signs are not specific, but can include coughing, nasal and ocular discharge, tachypnea, dyspnea, adventitious lung sounds, lethargy and (mild) fever (Pfützner and Sachse, 1996; Stipkovits et al., 2000; Caswell et al., 2010; Dudek et al., 2019). Chronic infections can result in decreased weight gain (Pfützner and Sachse, 1996; Stipkovits et al., 2000; Tschopp et al., 2001), and in severe cases, *M. bovis* can result in 5-10% mortality (Nicholas et al., 2008). Co-infection of *M. bovis* and BVDv may result in more chronic, unresponsive disease in veal calves and feedlots, and can aggravate clinical signs of *M. bovis* (Shahriar et al., 2002; Pardon et al., 2012).

Ultrasound can sometimes support the suspicion of a *M. bovis* infection, when consolidations and small abscesses are present (Fig. 2a). The pathological findings are exudative bronchopneumonia with caseous, suppurative or fibrinonecrotizing lesions, dark red areas of consolidation and pleuritis (Fig. 2b) (Rodriguez et al., 1996; Maeda et al., 2003; Radaelli et

al., 2008; Dudek et al., 2019; Haapala et al., 2019). Neutrophils constitute the major phagocytic cell type found in tissues or secretions in systems infected with *M. bovis* (Gagea et al., 2006; Maunsell et al., 2012).

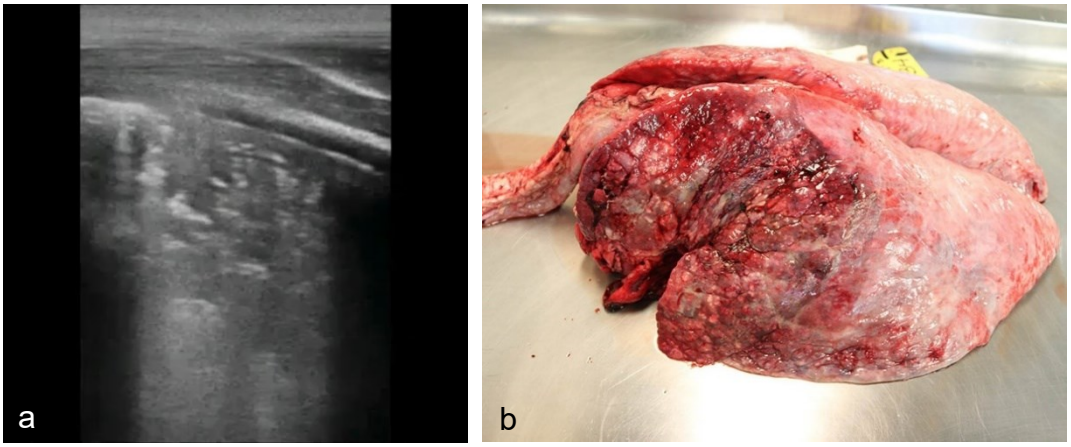


Figure 2. Ultrasound of calf with pneumonia caused by *M. bovis* showing consolidations and abcedation of the lung (a). Multifocal to coalescing necrosuppurative bronchointerstitial pneumonia caused by *M. bovis* (source: Noahs arkive, image F30248) (b).

Mastitis

Mastitis can either be present in a subclinical or clinical form in herds. When clinical signs are present, the non-responsiveness to treatment and the infection of several quarters with a milk drop and increased somatic cell counts are quite typical (González and Wilson, 2003; Al-Farha et al., 2017). The udder is sometimes swollen and red (Fig. 3a), and abscesses can develop. Milk consistency varies from mildly abnormal to gritty or purulent (Figure 3b) (Gonzalez and Wilson, 2003; Bokma et al., 2019b). Mastitis caused by *M. bovis* mostly occurs in dairy cattle, but can also affect beef cattle (Bokma et al., 2019b).

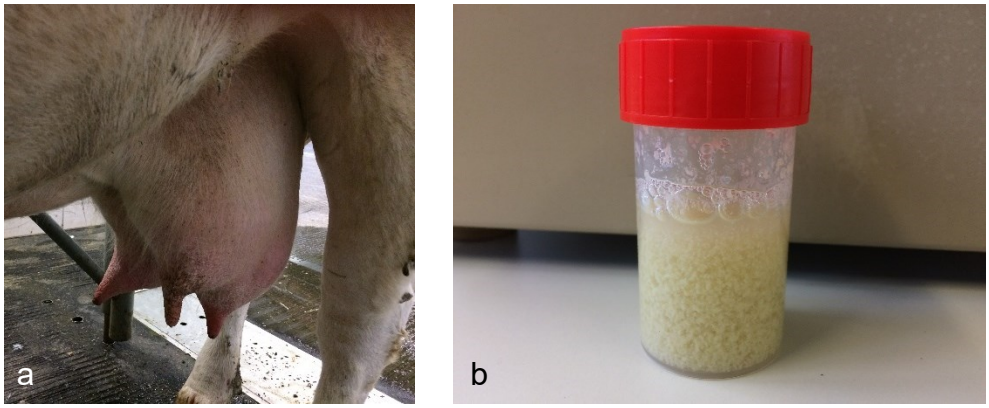


Figure 3. Clinical *M. bovis* mastitis in Belgian Blue beef cattle (a), and purulent milk obtained from this animal (b).

Otitis

Oral intake of *M. bovis* increases the risk of otitis in calves (Maunsell et al., 2012). Once *M. bovis* climbs into the middle ear via the Eustachian tube, it will easily multiply probably due to decreased reachability of the immune system (Maunsell et al., 2012). From the middle ear the infection can spread to the inner ear, and in severe cases even to the brain (Lamm et al., 2004). *M. bovis* has also been identified in axons of the facial nerves, but it is unclear how these got infected (Maeda et al., 2003). Clinical signs of otitis include typical head tilt (Fig. 4a), bilateral ear droop, purulent aural discharge (Fig. 4c) and neurological signs (Maeda et al., 2003; Francoz et al., 2004; Lamm et al., 2004; Nicholas et al., 2008).

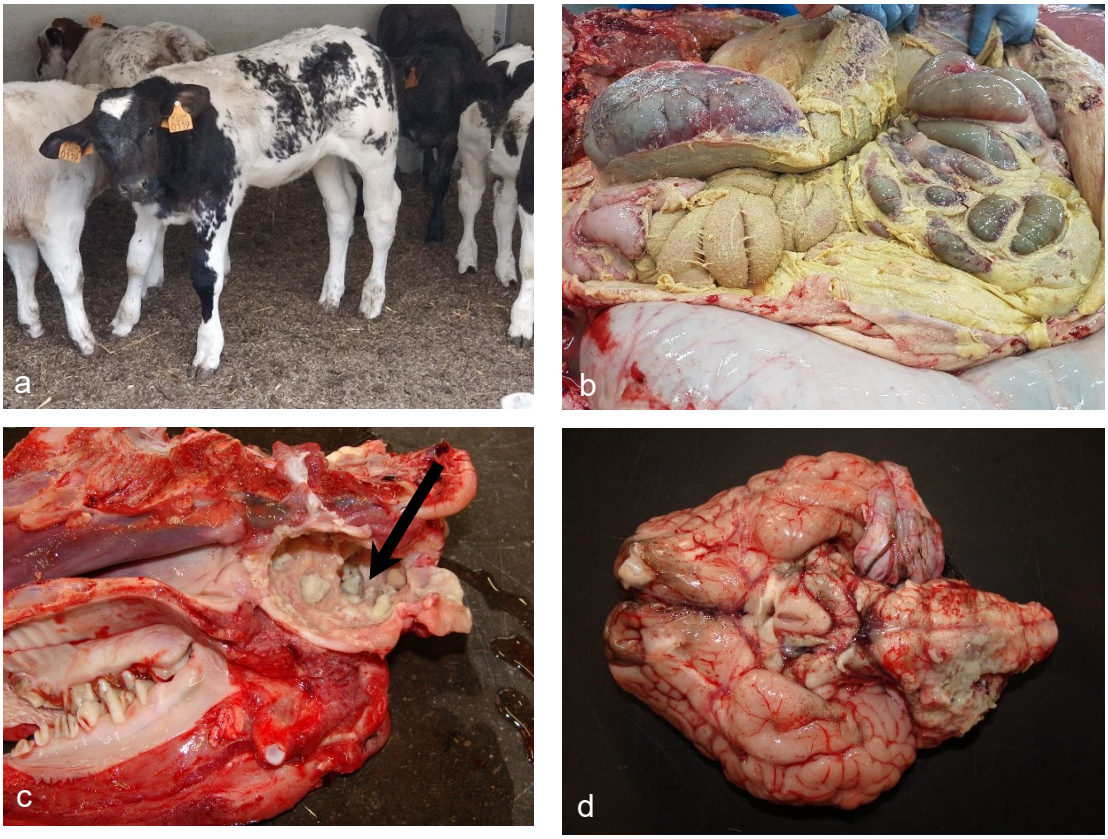


Figure 4. Typical head tilt in a calf with *M. bovis* otitis (a). Fibrinous omental bursitis and peritonitis caused by *M. bovis* (b). Skull of a calf with *M. bovis* otitis media and interna: purulent discharge in the tympanic bullae (c; arrow) (source: Bert De Jonge). Secondary purulent meningitis caused by chronic *M. bovis* otitis media (d) (source: Bert De Jonge).

Arthritis

Arthritis is commonly seen in both calves and cattle often together with pneumonia or mastitis (Stipkovits et al., 1993; Haines et al., 2001; Gagea et al., 2006; Wilson et al., 2007). Clinical signs are swelling of one or more joints, painful or warm joints, and lameness (Gagea et al., 2006; Wilson et al., 2007).

Less frequent manifestations of *M. bovis*

Next to the common *M. bovis* associated diseases, also others can occur, such as endocarditis (Kanda et al., 2019), myocarditis (Haines et al., 2004), seromas (Gille et al., 2016), peritonitis and bursitis (Fig. 4b) (Bokma et al., 2019b), meningitis (Figure 4d) (Stipkovits et al., 1993), keratoconjunctivitis (Levisohn et al., 2004; Alberti et al., 2006), genital disorders and abortion (Pfützner and Sachse, 1996; Hermeyer et al., 2012).

Epidemiology of *M. bovis* outbreaks

Once a herd is infected, *M. bovis* can very rapidly spread. In a study in four Pennsylvanian veal calf herds by Soehnlen et al. (2012), the *M. bovis* prevalence in nasal swabs went from 0% to approximately 85% within two months on farm. Also, in feedlot cattle and veal calves in other countries 51-100% of the animals seroconverted within a few weeks after exposure to *M. bovis* or became positive on culture and PCR (Stipkovits et al., 2001; Arcangioli et al., 2008; Wawegama et al., 2016; Becker et al., 2020). In dairy herds, *M. bovis* was shown to be highly contagious as well. Punyapornwithaya et al. (2011) showed that the presence of one *M. bovis* mastitis cow in the hospital pen could result in spread to 66% of the cows (6/9) within 12 days. The contagiousness of *M. bovis* was also supported by an experimental study in three cows that showed that inoculation with 10^8 colony forming units (CFU)/ml *M. bovis* in one quarter of the udder, resulted in a quick proliferation to 10^{10} CFU/ml in milk, and a spread to other quarters within 5 to 10 days. One cow was able to eliminate the infection, while the other two started shedding *M. bovis* again during the next lactation/after calving (Byrne et al., 2005). Intermittent shedding by carrier animals was also shown by Caswell and Archambault (2007), and is probably the reason for outbreaks of *M. bovis* in dry cows (Bicknell et al., 1983; Otter et al., 2015).

In the dairy sector *M. bovis* is mostly known as pathogen causing mastitis in adult cattle. However a recent Finnish study showed the importance to also give attention to calves and young stock (Vähänikkilä et al., 2019). The study showed the infection dynamics of the introduction of *M. bovis* in naïve herds. In 89.5% (17/19) of the infected herds, a few cases of

clinical mastitis occurred, mostly within 8 weeks after the index case. In 26.3% of the farms, positive PCR results on bulk tank milk (BTM) were found, but only in the first month after the index case. In 88.2% of these herds, *M. bovis* could be isolated from nasopharyngeal swabs from calves, and half a year later in 57.8% of these herds, calves were still positive. One and 1.5 year after the index case, *M. bovis* was still present in the calves on 47.4% and 31.6% of these herds, respectively (Vähänikkilä et al., 2019). However, whether this was an ongoing infection or a new introduction of *M. bovis* was not clear, as the *M. bovis* isolated at different time points were not compared by strain typing.

1.2 PREVENTION AND CONTROL

The contagious nature of *M. bovis* and the intermittent shedding by carrier animals emphasize the importance of prevention and control of this pathogen. Prevention signifies all measures taken to avoid a pathogen to enter a currently negative herd, whereas control stands for all measures taken to limit spread and consequences of an infection already present in a herd. It is important to understand risk factors for transmission and spread of *M. bovis* within and between herds to optimize both internal and external biosecurity. In the next chapters, risk factors for *M. bovis* entering into the herd and spreading within the herd will be outlined, as well as vaccination and treatment (both antimicrobial and anti-inflammatory drugs) possibilities.

Risk factors for *M. bovis* introduction into the herd

The most identified risk factors for introduction of *M. bovis* into the herd are purchase of animals from infected herds (Burnens et al., 1999; Bras et al., 2016; Pardon et al., 2020), and large herd size (Thomas et al., 1981; Fox et al., 2003; Murai and Higuchi, 2019). Herd size might also play a role in the clearance of clinical disease, as in Finland only small herds (< 70 animals) seem to be able to eliminate *M. bovis* compared to larger herds (Vähänikkilä et al., 2019). In contrast to previous studies, some authors did not find an association with herd size (Gonzalez et al., 1992; Gille et al., 2018a), which might at least partly depend on the cut-off used to define smaller and larger herd sizes and confounders, such as the presence of a breeding bull (Gille et al., 2018a). It is also likely that purchase and herd size are dependent factors, as purchase usually is necessary to expand the herd.

To give a better overview of current literature data, a meta-analysis was performed for purchase being a risk factor for *M. bovis*, showing that purchase was indeed associated with increased risk for a positive *M. bovis* herd, with a combined odds ratio of 5.91 (3.23-10.84) with a random effect model (Fig. 5). Heterogeneity is caused by random sampling error and true differences between studies (*e.g.* clinical diversity, methodological diversity). Here, heterogeneity might not be important as I^2 was below 30% and Chi^2 was not significant (P = 0.21). An attempt was made to equalize data to perform a meta-analysis and create a forrest plot for herd size as well. However, this attempt was unsuccessful since too many articles were inconclusive on herd size per farm to set a matching cut-off value between small and large farms or used it as continuous value. A weak point in several of the risk factor analysis studies is that only significant results are shown, therefore potentially biasing the meta-analysis. Also the diagnostic determination whether a herd is positive differed greatly between studies, as all kind of samples (nasal swabs, blood, bulk tank milk) and techniques were used (*e.g.* PCR, antibody ELISA, and combinations).

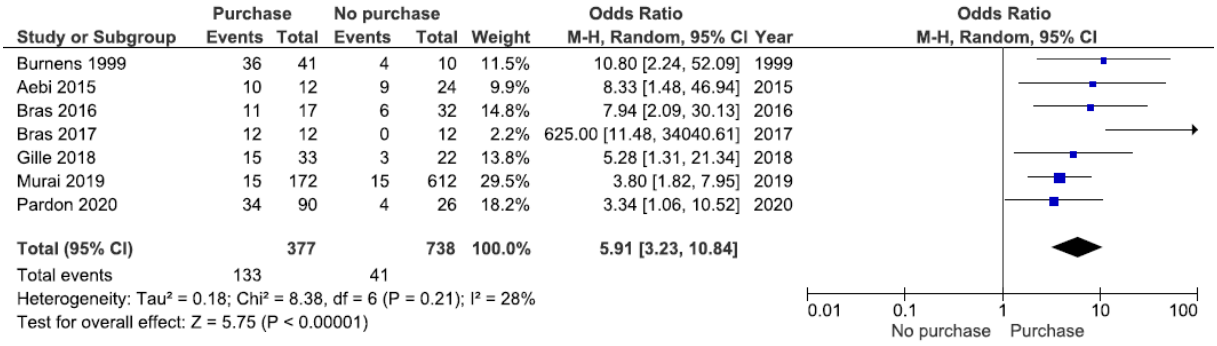


Figure 5. Forrest plot showing the meta-analysis of 7 published studies on purchase as a risk factor in *M. bovis* infections in cattle and bison herds. In Aebi et al. (2015), purchase also included sporadic transportations, expositions, and animal trade. The forrest plot was created with Review Manager 5.3.

Besides the above mentioned factors, other risk factors for introduction of *M. bovis* into the herd have been identified. One of the risk factors that is getting renewed attention is the transfer of *M. bovis* by semen, which has been demonstrated or suggested several times (Haapala et al., 2018; Yair et al., 2020). Also, herds with a breeding bull in the herd had 4.7 higher odds to be *M. bovis* positive in BTM than those without a breeding bull (Gille et al., 2018a). Exposure to a sale yard, animal movement, and using rental trailers or trailers from other farms can result in higher prevalence of *M. bovis* in cattle and bison farms (Aebi et al.,

2015; Bras et al., 2016; Schibrowski et al., 2018). Last, but not least, the transfer of *M. bovis* by fomites or persons has been identified as potential risk factor (Gonzalez et al., 1992). Gille et al. (2016) showed the onset of postsurgical seromas by a single *M. bovis* strain on different closed herds, while the veterinarian was the only visitor.

Risk factors for *M. bovis* spread within the herd

Once *M. bovis* is introduced into the herd, spread between individual animals can be continued by other factors. One of the main routes of transmission, is probably the colonization of calves by drinking *M. bovis* infected milk (Butler et al., 2000; Foster et al., 2009). The oral intake leads to colonization of the pharyngeal tonsils and an increased risk of pneumonia and otitis in calves (Maunsell et al., 2012). Nevertheless, Aebi et al. (2015) were not able to identify intake by milk as a risk factor. It was shown that colostrum can also contain *M. bovis*, although in low frequency. In Belgian colostrum samples of cattle from 17 different farms with an *M. bovis* outbreak within the last month were tested. Out of the 17 farms, only 4 were PCR positive for *M. bovis* (Gille et al., 2020). In another study however, colostrum fed by a tube was a risk factor in the univariable model, that was not withhold in the multivariable model (Gille et al., 2018a). Both observations are questioning the importance of *M. bovis* transmission by colostrum in comparison to the advantages for calves obtaining high quality colostrum. In Japan, the presence of an automatic calf feeder on farm was a risk factor for positive bulk tank milk samples (Murai and Higuchi, 2019).

The importance of airborne transmission is well recognized for *M. hyopneumoniae* in pigs and *Mycoplasma pneumoniae* in humans, but insufficiently evidenced to play a major role for *M. bovis* so far (Sánchez-Vargas and Gómez-Duarte, 2008; Otake et al., 2010). Only Jasper et al. (1974) were able to culture *M. bovis* on agar directly from air, while Soehnlen et al. (2012) were not able to repeat this. Whether disinfection of the barn is an important protective factor against *M. bovis* infections is also not clear. No risk factors associated with disinfection of the barn, the parcel around the barn or use of disinfecting footbaths could be determined so far (Murai and Higuchi, 2019). The importance of persistence of *M. bovis* in the environment for novel or reinfection in a herd is unclear. Nevertheless, Piccinini et al. (2015) were able to isolate *M. bovis* from the cages and mangers, which may be a risk for indirect transmission of *M. bovis* to other animals. Next to this, *M. bovis* can survive in straw and sand at 20°C for 10 days and 8 months, respectively (Pfützner, 1984; Justice-Allen et al., 2010). Another study showed survival for nearly 2 months in sponges and milk (4°C), and 2 weeks in water as well (Pfützner and Sachse, 1996). The latter might explain why that Schibrowski et al. (2018)

identified “shared water bucket” as risk factor for sero-increase between arrival and follow-up of Australian feeder cattle.

Other risk factors associated with housing are the absence of an individual calving pen (Gille et al., 2018a), the absence of a hospital pen (Fox, 2012), grouping of different age classes in one space (Tschopp et al., 2001), housing recently purchased animals in the same airspace (Pardon et al., 2020), stressors due to overcrowding and high in-barn temperature (Aebi et al., 2015). Also tie-stall in comparison to loose housing was a risk factor in the univariable model (Murai and Higuchi, 2019), although Burnens et al. (1999) were not able to find this association in a previous study.

Next to the above mentioned risk factors, management factors may also be associated with *M. bovis* positive herds. For example, no identification of attention cows with colour marks or leg tags to distinguish diseased cows from healthy cows increased the odds of isolating *Mycoplasma* species (mostly *M. bovis*) from the bulk tank milk (Pinho et al., 2013). In addition, corporation-type farms have a higher risk for a *M. bovis* positive herd, than family run farms (Murai and Higuchi, 2019).

A few important prevention measurements that can be implemented in the authors opinion and taking the above risk factors into account are: (1) identification of potentially infected or carrier animals when purchasing an animal and placing the animal in quarantine, (2) being aware of potential risk of breeding bull and artificial insemination, and (3) disinfection of barn, transport vehicles and fomites. For control, it is important to (1) terminate raw milk feeding, and (2) separate animals from different age groups, unknown health status and (chronically) infected animals. The role of vaccination and (metaphylactic) antimicrobial therapy in prevention and control will be outlined next.

Vaccination

In the last decades many attempts were made to develop vaccines against *M. bovis* (Perez-Casal et al., 2017; Dudek et al., 2021). Unfortunately until now, no commercial vaccines are available on the European market and only few vaccines are available in the United States. However, the efficacy of these vaccines has been questioned (Maunsell and Donovan, 2009; Nicholas et al., 2009; Soehnlen et al., 2011). The available vaccines are designed for use in healthy cattle aged 45 days (Pulmo-Guard TM MpB), 60 days (Myco-B one dose) or even four months (Myco-BAC® B), which is contradictory since most clinical signs occur in animals of two to six weeks of age (Stipkovits et al., 2000; Maunsell and Donovan, 2009). So

far, there are no reliable data showing that any (commercial) vaccine is working well enough for the use in young calves to prevent colonization or disease caused by *M. bovis* (Maunsell and Donovan, 2009; Perez-Casal et al., 2017).

There are many studies exploring the benefits of different vaccines in the prevention of *M. bovis* associated disease. However, all used a different challenge protocol (*e.g.* inoculation by aerosol, transthoracic or intravenously) and different concentrations and *M. bovis* strains (Chima et al., 1980; Nicolas et al., 2002). Published field studies often lack information concerning other pathogens causing BRD or control groups (Urbaneck et al., 2000; Dudek et al., 2016; Perez-Casal et al., 2017). Potential vaccine targets are for example the Vsps, conserved proteins, or membrane fractions of *M. bovis.* However, so far, attempts were not successful as the stimulated immune response did not result in protection against an experimental challenge (Nicholas et al., 2009; Mulongo et al., 2013; Prysliak et al., 2013; Prysliak and Perez-Casal, 2016). Another method that was explored, was the development of bacterins, which are mostly *M. bovis* strains inactivated by formalin or saponin treatment. This method was showing promising results, as the use of an autogenous vaccine containing formalin-inactivated strain of *M. bovis* and *M. haemolytica* resulted in the reduction of pneumonia in a feedlot (Urbaneck et al., 2000). The use of a vaccine that was inactivated with saponin resulted in less BRD lung lesions, lower rectal temperature compared to the unvaccinated group and less dissemination of *M. bovis* to other organs (Nicholas et al., 2002). Nevertheless, alternating results were obtained in cattle of different age, and in animals that were either infected before vaccination or not. Unfortunately, also aggravation of clinical BRD signs were observed in some vaccination studies (Rosenbusch, 1998; Bryson et al., 1999; Maunsell et al., 2009). In a blinded, controlled field trial in veal calves with two commercial bactericin vaccines in the USA (Pulmo-Guard^{TM} MpB, American Animal Health, Grand Priarie, Texas, USA and Mycomun® R, Biomune Co., Lenexa, Kansas, USA), a reduction in *M. bovis* lesions or colonization of the upper respiratory tract could not be attributed to vaccination (Soehnlen et al., 2011). In China, two *M. bovis* strains, attenuated by multiple passages (150 and 180), contributed to a significant reduction in lung lesions (Zhang et al., 2014). More recently three autogenous vaccines (inactivated with saponine) were administered to calves on three farms (two veal farms, one feedlot). After 6-9 months some differences were seen between vaccinated and unvaccinated calves in weight (higher, but not always significant in vaccinated calves), reduction of severe lung lesions and pleuritis (in

vaccinated calves), but there was no difference in the number of times that calves needed to be treated for BRD (Nicholas et al., 2019).

There are still many gaps in the development of vaccines for *M. bovis*, such as an optimized challenge protocol, the type of immune response needed for best protection, identification of the optimal adjuvant(s), identification of conserved surface antigens for subunit vaccines, and the mutation of individual genes in attenuated strains (Maunsell and Donovan, 2009; Dudek et al., 2016, 2018; Perez-Casal et al., 2017; Calcutt et al., 2018). In addition, even when it would be possible to import vaccines from the United States, there is a great possibility that those vaccines are based on *M. bovis* strains that differ from those circulating in Europe or even within a specific country (Kumar et al., 2020). Therefore, control is currently mostly depending on antimicrobial treatment and the isolation or culling of chronically infected animals.

1.3 ANTIMICROBIAL AND ANTI-INFLAMMATORY DRUG THERAPY

Most of the antimicrobial use in calves worldwide is to prevent or treat BRD (Dedonder and Apley, 2015; Lava et al., 2016; Lhermie et al., 2020), while the main indications to treat adult cattle with antimicrobials are acute mastitis and dry-cow therapy (Thomson et al., 2008; Kuipers et al., 2016). However, *M. bovis* mastitis is non-responsive to antimicrobial therapy (Pfützner, 1990; Nicholas et al., 2016). Therefore focus will be here on the treatment of BRD. BRD is a syndrome and can be caused by bacteria (*e.g. Pasteurellaceae*, *M. bovis*), viruses (*e.g.* bovine respiratory syncytial virus, BVDv, bovine herpes virus type 1, parainfluenza type 3 and bovine coronavirus), and environmental factors (*e.g.* housing, ventilation, ...), but most often a combination of those (Pardon and Buczinski, 2020). Diagnosis is necessary for targeted treatment, as clinical signs are often non-specific for any of the pathogens. Nevertheless, rapid treatment is often indicated and waiting for laboratory results can have disastrous consequences.

Prophylactic and metaphylactic antimicrobial use in BRD outbreaks

Mass medication or group treatment is widely used to prevent or treat BRD outbreaks, and has been shown to reduce BRD morbidity (Baptiste and Kyvsgaard, 2017). Treating healthy animals who are at risk with antimicrobials for the prevention of an outbreak is called ‘prophylaxis’, while ‘metaphylaxis’ is the treatment of animals that are not showing clinical

signs, but are in some way in contact with animals that are diseased and are expected to be infected (ECDC, 2015). In some studies, there is an additional threshold for the number of clinical cases within a group (*e.g.* 10%) or even for a certain amount of time (*e.g.* 2-3 days in a row) before it is defined as 'metaphylaxis' (Lees and Shojaee Aliabadi, 2002; Edwards, 2010). Although prophylactic use is forbidden in European countries (European Regulation 2019/6), metaphylaxis is still frequently used in different production systems. The largest effect of group treatment on morbidity reduction of BRD is observed when morbidity is ≥10% compared to contact with diseased animals or animals with fever (Baptiste and Kyvsgaard, 2017). Next to this, a meta-analysis on 58 publications showed that when the prevalence of BRD is low (< 25%), the 'number needed to treat' is high (7 calves) to prevent only one BRD case, which might not even be profitable in comparison to individual targeted treatment (Baptiste and Kyvsgaard, 2017). Only when morbidity is ≥50%, economic benefit is sometimes obtained (Baptiste and Kyvsgaard, 2017). However, to reduce antimicrobial use, the shift to individual treatment is necessary (Lava et al., 2016; Pardon, 2019). It was also shown that random clinical trials using parenteral antimicrobials alone resulted in lower morbidity than oral treatment alone (Baptiste and Kyvsgaard, 2017).

Despite the different opinions on whether metaphylaxis is necessary or not, taking into account that *M. bovis* is a primary pathogen, rapidly spreading due to its contagious nature, resulting in high morbidity, high antimicrobial use, hampered animal welfare, and economic losses, metaphylaxis in an *M. bovis* outbreak is perhaps justified. Nevertheless, before any treatment is started, diagnosis is necessary, as using an ineffective antibiotic as first intention treatment will only lead to the use of more antimicrobials, therapy failure, and economic loss.

Antimicrobial treatment of *M. bovis*

M. bovis is a member of the *Mollicutes* and lacks a cell wall (Razin, 1978). Therefore antimicrobials of which the working mechanism is based on damaging the cell wall, like beta-lactams (penicillines, cephalosporines) and polymyxins are not effective in the treatment of *M. bovis* associated diseases (Lysnyansky and Ayling, 2016). A second characteristic is the absence of folium acid production, with subsequent inherent resistance against trimethoprim and sulphonamides (Maunsell et al., 2011; Lysnyansky and Ayling, 2016). Intrinsic resistance against the 14-membered ring compound macrolide erythromycin is also described (Devriese and Haesebrouck, 1991; Francoz et al., 2005; Rosenbusch et al., 2005).

To treat *M. bovis* mainly florfenicol, tetracyclines, macrolides, and fluoroquinolones are used. Pleuromutilins are antibiotics which are not registered for the use in cattle, although they generally seem very efficient against *Mycoplasma* spp. in swine, and might be an option for use against *M. bovis,* as the use of critically important antibiotics (*e.g.* macrolides, fluoroquinolones) are discouraged by the World Health Organization (WHO) and the World Organization for Animal Health (OIE) (Aidara-Kane et al., 2018; OIE, 2019). In multiple European countries, including Belgium, the use of fluoroquinolones without a susceptibility test showing it to be the last resort therapy is even prohibited (KB 21 July 2016).

Several authors have attempted to investigate antimicrobial treatment protocols in experimental studies or clinical field trials. Unfortunately, there are many pitfalls in the interpretation of effectiveness, such as influence of different study populations and co-infections with other pathogens. Clinical field trials are also costly, time-consuming, and ethical reasons (*e.g.* inclusion of a negative control group) might prevent such studies from happening frequently. In addition, the used *M. bovis* strain might not always reflect the pathogenicity and antimicrobial susceptibility of other *M. bovis* strains. Next to different pathogenicity, there are no standard protocols for the determination of MIC values. Described MIC values of experimental strains should therefore be interpreted with caution.

When using antimicrobials as prophylaxis for BRD, best relative risk reductions ('the difference in event rates between two groups, expressed as a proportion of the event rate in the untreated group', UWA, 2021) were shown when using broad-spectrum critically important antimicrobials (fluoroquinolones, macrolides) or combinations (tetracycline-combinations). However, when using different antimicrobials as metaphylaxis instead of prophylaxis, no significant difference on morbidity outcome between antimicrobials was identified (Baptiste and Kyvsgaard, 2017). In the next paragraphs, a selection of clinical trials evaluating antimicrobial treatment protocols for *M. bovis* is briefly discussed.

Florfenicol and tetracyclines

In 2008 in a veal setting where *M. bovis* was the dominant pathogen, Catry et al. (2008) showed that the use of parenteral florfenicol was superior compared to the oral use of tilmicosin, doxycycline or saline. Less calves that were initially unaffected got sick, and an increase in body weight, and decrease in rectal temperature was observed (Catry et al., 2008). In addition, in a feedlot setting, Hendrick et al. (2013) showed that prophylactic treatment

with oxytetracycline compared to no treatment resulted in a reduced risk of BRD, but an increased risk for arthritis. However, no significant effect on BRD relapse, average daily gain or mortality was observed. In another study, in a natural outbreak of BRD including *M. bovis*, no significant differences were observed on the improvement of clinical signs, body weight gain or relapse within 60 days, when cattle were individually treated with either florfenicol or tulathromycin (Godinho et al., 2005b). In a recent natural outbreak of *M. bovis* pneumonia in beef young stock, treatment with florfenicol or oxytetracycline resulted in cure rates of 94.3% and 85.7% after 8 days, respectively, as evidenced by thoracic ultrasonography (De Cremer et al., 2019). These studies show that florfenicol and oxytetracycline can probably both be used as first intention parenteral treatment in BRD outbreaks. To what extent, acquired resistance to these molecules is frequent will be discussed later in this literature overview.

Macrolides

After prophylactic tilmicosin treatment in gnotobiotic calves the colonization of *M. bovis* was reduced, but not prevented (Gourlay et al., 1989). Also, treatment upon presence of clinical signs, reduced clinical scores and lung lesions. However, in both experimental and natural outbreaks, the superiority of tulathromycin compared to tildipirosin and tilmicosin as treatment for calves infected with *M. bovis* (MIC >64 µg/ml for tulathromycin) was shown by reduced depressed position, lower rectal temperatures, reduced lung lesions, reduced mortality, and a higher body weight (Godinho et al., 2005b; Bartram et al., 2016). In a controlled field trial where *M. bovis* (MIC > 16 µg/ml), *M. haemolytica* (0.5-2.0 µg/ml), and *P. multocida* (0.125-2.0 µg/ml) were involved in BRD, both the morbidity and the number of animals showing clinical signs in the first 14 days after treatment were significantly lower in the group treated with gamithromycin compared to saline (Baggott et al., 2011). Based on these studies, which were often executed or sponsored by the manufacturer of the product, tulathromycin and gamithromycin appear very efficient in the treatment of *M. bovis*. It is however concerning, that 14 days post-treatment *M. bovis* was still present in the lungs (Godinho et al., 2005a; Bartram et al., 2016). It would have been interesting in these studies to prolong the sampling time until slaughter, and to determine whether animals would have been able to cure themselves and eliminate *M. bovis* fully after a longer period or whether they keep shedding *M. bovis* and are able to (re)infect other animals.

Spectinomycin

Another antimicrobial showing to be potentially effective in the treatment of *M. bovis* in 8 experimentally infected calves by intratracheal inoculation was spectinomycin (MIC 4.2 ± 2 mg/L) (Poumarat et al., 2001). Lower concentrations of *M. bovis* were observed in the lung lobes after slaughter compared to those who were not treated. Unfortunately, *M. bovis* was still present in the lungs after treatment. The duration of the therapy in this trial (only 3 days) might have contributed to the fact that treatment did not result in clearance of the bacterium from the lungs, since it was recommended to treat animals for at least 10 days with antimicrobials, as otherwise relapse (30-70% of the animals) might occur (Currin, 2009).

Valnemulin

As mentioned before, pleuromutilins are not registered for antimicrobial therapy in cattle. Nevertheless there are studies showing promising results for valnemulin. A clinical field trial with *M. bovis* was executed with valnemulin in feed (21 days) compared to a placebo premix in a single farm. The results showed a decrease in *M. bovis* associated disease signs, reduced treatment incidence and mortality, and increased weight (Stipkovits et al., 2001). In addition, an experimental study was performed, where calves were treated with either enrofloxacin or valnemulin in the milk replacer (10 days) or they were not treated after infection (Stipkovits et al., 2005). Clinical score reduced significantly more rapid in the valnemulin group compared to the enrofloxacin and the group that was infected, but not treated. Rectal temperature did however reduce in both the valnemulin and enrofloxacin group without significant difference between these two. Isolation of *M. bovis* in different organs after slaughter showed significantly less isolation of *M. bovis* from animals treated with valnemulin than those with enrofloxacin (Stipkovits et al., 2005). Parenteral treatment of valnemulin and tiamulin, which are products available in swine, could therefore be further explored for the individual treatment of cattle infected with *M. bovis*.

Combination of antimicrobial treatment and anti-inflammatory drugs

More and more anti-inflammatory drugs (AIDs), especially non-steroidal anti-inflammatory drugs (NSAIDs), are used when animals are combatting an infectious disease for the reduction of fever, to relieve pain, and prevent the potential collateral damage caused by an overreaction of the immune system (Leslie and Petersson-Wolfe, 2012; Thiry et al., 2014). It is however not clear, whether this use of anti-inflammatory drugs, is recommended in *M. bovis* treatment.

A systematic review on evidence related use of anti-inflammatory drugs in BRD was performed by Francoz et al. (2012), showing at that time only 15 articles meeting the inclusion criteria. All differing greatly on study population, duration, and used (NS)AIDs. The only beneficial effect most of the randomized and blinded studies (n=6) were clear on was the rapid decrease in rectal temperature (Francoz et al., 2012). Additional potential NSAID benefit was the decrease in lung consolidations, although no improvement in clinical parameters was observed (Francoz et al., 2012). None of these six studies mentioned the presence of *M. bovis*.

Since this systematic review, several studies were published including *M. bovis* as pathogen, and indeed rectal temperature, but also the depression score reduced significantly after six hours of administration of florfenicol-flunixin in comparison to florfenicol alone, when this was given to calves showing BRD signs. In this study, beside *M. bovis*, also other pathogens were present (Thiry et al., 2014). Next to rectal temperature, also respiratory signs reduced, although there was no significant difference between florfenicol alone or in combination with the NSAID. Limitation of such a study is that all BRD pathogens are observed together, and it is not clear whether *M. bovis* would respond different. In Dudek et al. (2019) the efficacy of either enrofloxacin treatment alone (SC, 3 days), or in combination with flunixin meglumine (NSAID, intravenously (IV), 3 days), when calves were experimentally inoculated with *M. bovis* (MIC enrofloxacin 0.25 μg/mL) was explored. A third treatment option was the combination of both enrofloxacin, flunixin meglumine, and the immunostimulator pegbovigrastim (day 1 and 7, SC). Also a negative control group was included. The best immune response, the least lung lesions and clinical signs were observed in the group treated with only enrofloxacin, while the combination of enrofloxacin with the NSAID or with the NSAID and immunomodulator showed more lesions.

Another AIDs class are the corticosteroids. When 20 Jersey calves were experimentally infected with *M. bovis* in their milk, it was shown that the treatment with dexamethasone (IM, day 2) increased shedding of *M. bovis* in treated calves compared to those who were not treated. Next to this, the onset of shedding occurred faster and the duration of *M. bovis* shedding prolonged, suggesting that stress will also increase shedding (Alabdullah et al., 2017). Another study suggested the potential synergistic influence of dexamethasone and *M. bovis* on reduced neutrophil functioning (Alabdullah et al., 2015), while same authors showed the impaired phagocytizing number of *M. bovis* by neutrophils exposed to dexamethasone

treatment (Alabdullah et al., 2018). Therefore, use of corticosteroids in *M. bovis* infections should be discouraged.

Therapy evaluation and failure of treatment

When facing an outbreak of BRD in calves, first one should make the decision whether an animal (or group of animals) needs to be treated (= antimicrobial decision making process). Often, this is based on the detection of clinical signs or pneumonia on ultrasound. Usually, samples for the detection of the pathogen are taken (nasal or lung swabs, bronchoalveolar lavage, transtracheal aspiration), and while pending laboratory results, empiric treatment, based on experience and available, local antimicrobial susceptibility information, is initiated. Depending on the turnaround time of the diagnostic tests and the severity of the disease, therapy success is clinically evaluated or identification of the pathogen and antimicrobial susceptibility testing will show whether an (in)appropriate antimicrobial treatment was started (Pardon, 2019). Inappropriate antimicrobial treatment is defined as: "The use of antibiotics with poor or no *in vitro* activity against the identified microorganisms causing infection at the tissue site of infection" (American Thoracic Society and Infectious Diseases Society of America, 2005). Whether *in vitro* includes translating *in vitro* results to *in vivo* results with clinical breakpoints is not clear, but these data should be included as well. In addition to 'appropriate treatment', the treatment should also be 'rational', which is defined as: "patients receive medications appropriate to their clinical needs, in doses that meet their own individual requirements, for an adequate period of time, and at the lowest cost to them and their community" (WHO, 1985). The cost to their community, in terms of consequences, is especially important concerning the consequences of antimicrobial use, such as antimicrobial resistance. Therefore, the term 'prudent use' is also often applied: "Use which benefits the patient while at the same time minimizing the probability of adverse effects for the individual and the emergence or spread of antimicrobial resistance" (Pardon, 2019).

Unfortunately, even when the laboratory results show that the appropriate antimicrobial treatment was initiated, therapy failure can still occur (or vice versa). Therefore, the 'definitive therapy' should be decided while considering both clinical outcome and laboratory results. The pharmacokinetics and dynamics of certain antimicrobials are unpredictable in diseased animals. For example, florfenicol and marbofloxacin pharmacodynamics vary in diseased sheep and calves compared to healthy animals (Ismail and El-Kattan, 2007; Pérez et al., 2015; Booker and Lubbers, 2020). Next to this, medication errors ("any preventable event

that may cause or lead to inappropriate medication use or patient harm while the medication is in the control of the health care professional, patient, or consumer", NCCMERP, 2021), such as with stocking temperatures, administration route, dose, and frequency of treatment, can result in treatment failure (Booker et al., 2020). For *M. bovis,* treating early in the disease course is of great importance as well (Currin, 2009), due to its immunomodulatory character, and formation of abscesses, giving it the ability to evade antimicrobials and the immune response. Delayed treatment even with the 'appropriate' antimicrobial treatment, can therefore result in therapy failure.

In conclusion, the consequences of *M. bovis* outbreaks are extensive of which economic losses, high antimicrobial use, decreased defense mechanisms against other pathogens, and hampered animal welfare are only a few. Rapid identification of *M. bovis* and obtaining its susceptibility profile, is absolutely crucial for improved control and prevention. Next to rapid identification and susceptibility testing, strain typing can help to better understand possible introduction pathways and the epidemiological behavior of *M. bovis*. Both can help in the development of additional (inter)national guidelines for prevention and control of *M. bovis.*

In the following chapters of this general introduction, the available diagnostic methods for identification, strain typing, and antimicrobial susceptibility testing will be discussed. Additionally, a brief overview of the available epidemiological data obtained by these methods will be given.

1.4 IDENTIFICATION OF *MYCOPLASMA BOVIS*

When facing a BRD outbreak, it is very important to know whether *M. bovis* is one of the involved agents, as first choice antimicrobial therapies for BRD often include antimicrobials that are not effective against *M. bovis*. For example, in the Netherlands (NL) and Belgium (BEL) the guidelines for antimicrobials use in BRD outbreaks include penicillin and potentiated sulphonamides, while for BRD including *M. bovis*, the first choice agents include florfenicol (NL, BEL), tetracyclines (NL) and macrolides (NL). In addition, as previously touched upon, metaphylactic treatment might be justified in the treatment of *M. bovis*. Therefore, to enable immediate rational use of an appropriate antimicrobial resulting in more therapy success and better control of further spread, early identification of the presence of *M. bovis* is crucial.

Identification of *M. bovis* faces several challenges. Although many methods are available to identify *M. bovis,* none of them are perfect. Many can be improved, especially in terms of turnaround time, costs, and diagnostic accuracy. For instance, culture takes about 1-2 weeks to obtain definitive identification of *M. bovis*, costs of (multiplex) PCR can easily amount to 125 euros per sample, and sensitivity of antibody ELISA is often disappointing. Most samples investigated for *M. bovis* are either from the respiratory tract, milk or blood for serology. Occasionally synovial samples, swabs from the middle ear, reproductive tract or other organs are presented. Conventional techniques for routine diagnostics/identification of *M. bovis* can be divided into two large categories: (1) detection of the bacterium (*e.g.* microbial culture, PCR) or (2) detection of antibodies (*e.g.* ELISA), showing a previous, but not necessarily present, infection with *M. bovis*. Detection of the bacterium can be subdivided into (a) culture-based methods resulting in an isolate, and (b) culture-independent methods (*e.g.* PCR), without obtaining an isolate from the sample (Parker et al., 2018).

In the last decade, MALDI-TOF MS (Matrix-assisted laser desorption-ionization – time of flight mass spectrometry), has become a technique for faster diagnosis of bacteria following a cultivation step (Bizzini and Greub, 2010; Puchalski et al., 2016), including *Mycoplasma* species (Pereyre et al., 2013). Also efforts to make next-generation sequencing, a culture-independent method, more accessible and affordable for diagnosis and strain typing have been made (Parker et al., 2018).

Sampling methods in the standing animal

In a BRD outbreak different sampling methods can be executed by the veterinarian. Most often a deep nasal swab (DNS; Fig 6A), a bronchoalveolar lavage (BAL; Fig 6B) or a trans-tracheal aspirate/wash (TTA/TTW; Fig 6C) is performed. In short, before a DNS or BAL is performed the nostrils are disinfected with alcohol (70-90%). To perform the DNS, a swab is introduced via the nasal cavity until the nasopharynx where the swab is rotated, resulting in a sample of the nasopharyngeal mucosa (Van Driessche et al., 2017). In case of a BAL, a small sterilized catheter is introduced in the nasal cavity, carefully moved up passing the larynx and through the trachea until the bronchi are reached. Subsequently approximately 30 mL of saline is injected via the catheter, and immediately aspirated (Van Driessche et al., 2017). In this case an individual lung lobe is sampled (Pardon and Buczinski, 2020). For a TTA or TTW first the skin should be shaved, sterilized, and a local anesthetic should be applied at the lower part of the trachea. Subsequently, a human intravenous catheter or Large Animal Trans-Tracheal Wash Kit (MILA international) is used to push through the skin into the trachea

where saline can be injected. The saline washes the tracheal bifurcation, and can be aspirated for further analysis (Timsit et al., 2013; Pardon and Buczinski, 2020).

Figure 6. Methods to sample the respiratory tract of cattle: deep nasal swab (a), bronchoalveolar lavage (b), and trans-tracheal aspirate/wash (c). Source: Pardon and Buczinski, 2020.

These techniques all have their advantages and disadvantages considering ease of use, cost, interpretation of test outcome, invasiveness and possible complications which are summarized in Table 1, and extensively described elsewhere (Pardon and Buczinski, 2020). Important for the interpretation of test results is the difference between isolating or identifying a pathogen from the upper respiratory tract (DNS) or the lower respiratory tract (the larger bronchial airways and alveoli) with a BAL (Chase and Kaushik, 2019). Some opportunistic pathogens (*e.g. Pasteurellaceae*), can be present on the nasopharyngeal mucosa as part of the common flora, not associated with pneumonia. However, when isolating them abundantly from the lung, they are most likely involved in pneumonia, and antimicrobial treatment is necessary. Opinions on the reflection of pathogens isolated from TTA/TTW are somewhat controversial, as some believe that only pathogens from the lung are transferred by cilia towards the bifurcation, while others comment on the possibility of aspirating mucus originating from the nasopharynx or indicating a bacterial tracheitis without the need of antimicrobial therapy (Pardon and Buczinski, 2020). In addition, DNS are resulting in more polymicrobial cultures (~80%) than those from BAL (~20%), which are more difficult to interpret (Van Driessche et al., 2017). However, it was shown by Doyle et al. (2017) that the agreement between DNS and BAL compared to TTW was good to very good for most pathogens involved in BRD, although the agreement for *M. bovis* was highest in BALf, and more *M. bovis* was isolated from BALf than TTW.

Table 1. Sampling methods for the identification of *M. bovis* in cattle

Sampling method	Invasiveness	Easiness	Culture *M. bovis*	Clinical Interpretation	Cost	Sampling Time***
(Deep nasal) swab*	±	+	±	±	€	1m
Trans trachael aspirate/wash	++	±	+	+	€€	10m
Bronchoalveolar lavage	+	±	+	+	€€	1-10m
Milk	-	+	+	+	€	1m
Body fluids**	±	±	+	+	€€	5-10m
Blood (serology)	±	+	-	±	€	1m

* swabs can also be from the middle ear, eye or reproductive tract
** *e.g.* joint, pleural, abdominal or cerebrospinal fluid
*** sampling time in minutes (m), can vary between veterinarians depending on experience

Other sampling methods, such as milk samples and body fluids, can be implemented in *M. bovis* mastitis or arthritis outbreaks, and problems with postsurgical seromas. Retrieving reliable milk samples rely on the correct sample technique (*e.g.* cleaning and disinfecting the teat, forestripping, handling the sterile sample tube, ..). Also, puncturing the skin to obtain body fluids, asks for a correct disinfection protocol to prevent contamination of the sample and introduction of an (new) infection in the body fluid.

Culture-based methods for the identification of *M. bovis*

First of all, sample storage is a key factor for the isolation of *M. bovis* from clinical samples. Not only because of potential overgrowth by other pathogens or contaminants, but also due to the fact that *M. bovis* does not survive very well in most samples. For example, *M. bovis* shows only short survival in milk at room temperature (23ºC) compared to storage of the samples in the fridge (5ºC) (Parker et al., 2016). It is recommended to store samples at 5ºC and culture them as soon as possible. After one day of storage, rapid reduction of viable *M. bovis* is observed (Boonyayatra et al., 2010). When delayed culturing is foreseen, samples can be frozen, as only a reduction of 1-2 $\log_{10}$ in viable *M. bovis* is observed in milk and colostrum samples (Biddle et al., 2004; Boonyayatra et al., 2010; Vyletělová, 2010; Gille et al., 2018b). Addition of glycerol (10-30%) can improve recovery from frozen milk samples (Boonyayatra et al., 2010).

Conventional microbial culture

Mycoplasmata require specific solid or liquid media to grow and interpretation needs a certain amount of expertise (Parker et al., 2018; Wisselink et al., 2019). The limit of detection of *M. bovis* culture is dependent on the exact method used, but has been estimated around 10^2 CFU/mL in milk and in BALf (Sachse et al., 2010; Wisselink et al., 2019). Advantages of

culturing are simultaneous detection of other *Mycoplasma* species, low operating costs, the acquirement of an isolate, with possibility for additional molecular typing and antimicrobial susceptibility testing (AST), and a more straightforward clinical interpretation, as viable organisms are detected. Disadvantages are the long turnaround time before final identification is obtained (5-10 days), potential overgrowth by other pathogens or contaminants, and the need for correct sample preservation to safeguard the viability of the organisms. When cultured on solid media and inspected under the microscope, *M. bovis* shows a "fried egg" morphology, which is quite specific for the *Mycoplasma* genus, but not specific for *M. bovis*. Therefore, most culture-dependent systems require species confirmation using PCR or 16S rRNA sequencing, which is expensive and requires additional time. Confirmation after culture can also be obtained with a commercially available sandwich ELISA, which is applicable to tissue lysates and milk (Bio K 341, Bio X). It has also been described that *Mycoplasma* species can be distinguished with biochemical tests, such as film production and metabolic inhibition tests (Poveda and Nicholas, 1998). For example digitonin or nisin disc diffusion assays can be used to distinguish *Mycoplasma* from *Acholeplasma* species. To identify *M. bovis*, the presence of lipase activity can be tested with a selective-indicative agar containing Tween-80 (Devriese and Haesebrouck, 1991; Boonyayatra et al., 2012). Because such biochemical tests may not be very specific, it is unfortunate that diagnostic accuracy of these methods is poorly described until now.

MALDI-TOF MS: novel culture-based method

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a technique which has become very popular in veterinary diagnostics over the last few years (Randall et al., 2015). Whereas the purchase of this device is a great investment, today many laboratories already have their own MALDI-TOF MS equipment. The easiness of the 'direct transfer method', where colonies of bacteria from solid medium are deposited on target plates, covered with matrix, and are immediately ready for analysis, contributes to its popularity. Samples are vaporized and ionized without degradation due to the matrix by a laser, and the particles are subsequently accelerated in an electric field. Depending on their mass and charge, it takes a certain amount of time to arrive at the detector (large particles are slower) (Pavlovic et al., 2013; Bourassa and Butler-Wu, 2015), and the detection of the most abundant proteins, results in species-specific spectra. This spectrum can be compared to a commercial and/or in-house library (Sauer and Kliem, 2010; Pavlovic et al., 2013).

MALDI-TOF MS is mostly detecting proteins, which are usually unique per bacterial species (Clark et al., 2013; Dingle and Butler-Wu, 2013). Therefore, MALDI-TOF MS is even able to distinguish *M. bovis* from *M. agalactiae* better than 16S rRNA sequencing or species-specific qPCR (Pereyre et al., 2013; Cornelissen et al., 2017; Spergser et al., 2019). However, the proteome of microorganisms, and therefore the quality and reproducibility of spectra, can be influenced by different circumstances, such as growth medium and incubation time (Welker and Moore, 2011; Anderson et al., 2012; Martiny et al., 2013). Another influence on the results can be caused by the MALDI-TOF MS device that is used. Both Bruker Biotyper (Bruker Daltonics) and VITEK MS^{TM} PLUS (bioMérieux) show a high level of agreement, although the commercial libraries are not completely the same (TeKippe and Burnham, 2014; Lévesque et al., 2015). It was shown that the VITEK MS result in more misidentifications when the bacteria of interest is not part of the library (Lévesque et al., 2015). Nevertheless, these libraries are frequently updated improving both devices.

For *Mycoplasma* species, the 'direct transfer method' does not seem sufficiently reliable, due to the small colonies and often growth into the agar. Nevertheless, MALDI-TOF MS is able to perform identification of *Mycoplasma* species after an enrichment step and protein extraction from liquid culture (Pereyre et al., 2013; Spergser et al., 2019).

MALDI-TOF MS is easy to use, rapid and compared to PCR, the consumable cost is much lower. Nevertheless, until now it remains unclear what the best growth conditions are allowing the fastest and most reliable identification of *M. bovis* for clinical applications.

Culture-independent methods for the identification of *M. bovis*

When isolation of *M. bovis* is not possible as a result of for example chronically infected organs or previous antimicrobial treatment, *M. bovis* can be visualized with the use of monoclonal antibodies (immunohistochemistry) or labeled DNA (in-situ hybridization) (Adegboye et al., 1995; Hermeyer et al., 2012). However, these methods are costly and require time and experienced staff (Dudek et al., 2020). In contrast, PCR has become the routine diagnostic method to identify *M. bovis*, as it is much faster, and will therefore be further outlined beneath.

Conventional and real-time PCR

PCR is a technique that detects specific parts of the DNA, and amplifies this part of the DNA until it can be visualized by fluorescent detection. One of the advantages of PCR is that storage conditions are less relevant than with culture-based methods, as DNA is the target and

no preceding culturing step is required (Parker et al., 2018). Both conventional (cPCR) and quantitative (real-time) PCR (qPCR) are frequently used in the detection of *M. bovis*. The advantage of qPCR compared to cPCR is a faster turnaround time. In addition, quantification of the sample content is possible with qPCR (Maurin, 2012). In general, for both cPCR and qPCR, a final identification can be reached more rapidly than with culture-dependent methods. The limit of detection is lowest in milk (10^2 CFU/ml) and BALf (10-10^3 CFU/ml), followed by semen (10^5 CFU/ml) and different kind of swabs (preputial, vaginal, eye and nasal) (10^6 CFU/ml). Besides the sample, the detection limit also depends on the targeted gene (respective sensitivity: *uvrC* > *oppD* > *polC*) (Parker et al., 2017; Wisselink et al., 2019), and the number of species in one sample (more species increases detection limit) (Parker et al., 2017). Many different methods for *M. bovis* qPCR are applied, not only using different target genes, such as the *oppD*, *uvrC*, *fusA* and *polC*, and 16S rRNA, but also different DNA extraction protocols and amplification platforms are used (Wisselink et al., 2019). Nevertheless, despite the multiplicity of cPCR and qPCR methods for *M. bovis* used in different laboratories, an interlaboratory evaluation showed comparable performance of all tested PCR methods (Wisselink et al., 2019). For one of these (triplex) real-time PCR tests sensitivity was 95.2% and specificity 73.9% for identification of *M. bovis* from BALf compared to PCR with denaturing gradient gel electrophoresis fingerprinting (PCR/DGGE) (Cornelissen et al., 2017). Another study showed comparable results for Pneumo4B multiplex qPCR kit on tracheal aspirates, as sensitivity and specificity were 96% and 71%, respectively (Pansri et al., 2020). Disadvantages are the same for cPCR as qPCR, such as higher costs compared to culture, other *Mycoplasma* species not being detected (except with multiplex tests, additional denaturing gradient gel electrophoresis (DGGE), or high resolution melting curve analysis (HRM)), possible cross-contamination of samples in the laboratory resulting in false positives, cross-reactivity with other *Mycoplasma* species (especially *M. agalactiae*), and a less straightforward clinical interpretation, because also non-viable organisms can be detected. In addition, no isolate will be obtained, and no strain database can be created. Therefore, the possibility for additional studies on antimicrobial susceptibility or strain typing are, although not completely non-existent (*e.g.* MLVA after PCR), very limited (Pinho et al., 2012).

Potential culture-independent *M. bovis* identification methods

Lately, some other promising, but not routinely used methods were also published. For example the Procedure for Ultra Rapid Extraction and Loop-mediated isothermal

Amplification (PURE-LAMP) by Itoh et al. (2020). This method can identify *M. bovis* from milk within 2 hours and showed high sensitivity (85-97%) and specificity (100%) on individual milk samples (Itoh et al., 2020). Another point-of-care diagnostic method is the Rapid Polymerase Amplification Lateral Flow Dipstick (RPA-LFD), which shows the result within 30 minutes from different kind of samples (nasal swabs, joint, bulk tank milk, ..) with a sensitivity and specificity of 99.0% and 95.6%, respectively. There is no need for expensive equipment, and the interpretation is easy (Zhao et al., 2018). Finally, high resolution melting curve analysis (HRM), seems like a potential method for the identification of *M. bovis* (Ahani Azari et al., 2020). Nevertheless, more research is necessary to validate the above mentioned methods, before they can be implemented as reliable routine diagnostic methods.

Next-generation sequencing: an upcoming culture-independent method

Due to fast decreasing cost for whole genome sequencing (WGS) and decrease in turnaround time, it is becoming more attractive as an all-in-one diagnostic tool (*e.g.* identification, strain typing and AST). Sanger sequencing was the first-generation sequencing method, based on a single DNA fragment amplification at the time, resulting in short (800-1000 base pairs), but very accurate sequences. The second-generation sequencing is most commonly referred to by the Illumina short-read technology. Illumina technology is based on the sequencing-by-synthesis approach resulting in even shorter reads (150-250 base pairs), but is in contrast to Sanger, able to sequence millions of DNA fragments at the same time (Goodwin et al., 2016). The many obtained short-reads can be a problem for *Mycoplasma* species due to their highly repetitive regions and extreme low GC content. Therefore, difficulties can occur during the reconstruction of a consensus genome, as with many small fragments it is hard to determine the exact location of the DNA fragment and how many repetitive regions are exactly present at a specific location (Sabat et al., 2013).

However, third generation sequencing, such as technologies provided by Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT), enable the real-time sequencing of longer reads (20-200 kb), making the reconstruction of a consensus genome more easy in general and for *Mycoplasma* species in specific.

PacBio's Single Molecule Real-Time (SMRT) sequencing uses a SMRT flow cell with zero-mode waveguides. When nucleotides are incorporated by a modified polymerase, the detection of phosphor-linked fluorophores allows to capture real-time incorporation of nucleotides (Slatko et al., 2018; https://www.youtube.com/watch?v=_lD8JyAbwEo&t=1s). A

big difference between nanopore sequencing and other platforms is the absence of an amplification step supported by polymerase with nanopore sequencing. Instead of detecting a secondary signal, nanopore sequencing directly detects ssDNA which can be bidirectional sequenced due to the addition of a hairpin (Goodwin et al., 2016). The DNA is passed along small channels (nanopores) (Fig. 7A), detecting k-mer-specific electrical currents (squiggles), which eventually allows interpretation of the k-mer sequence on the dsDNA strand (Fig. 7B) (Slatko et al., 2018; https://www.youtube.com/watch?v=GUb1TZvMWsw). A brief comparison between second and third generation sequencing methods is shown in Table 2.

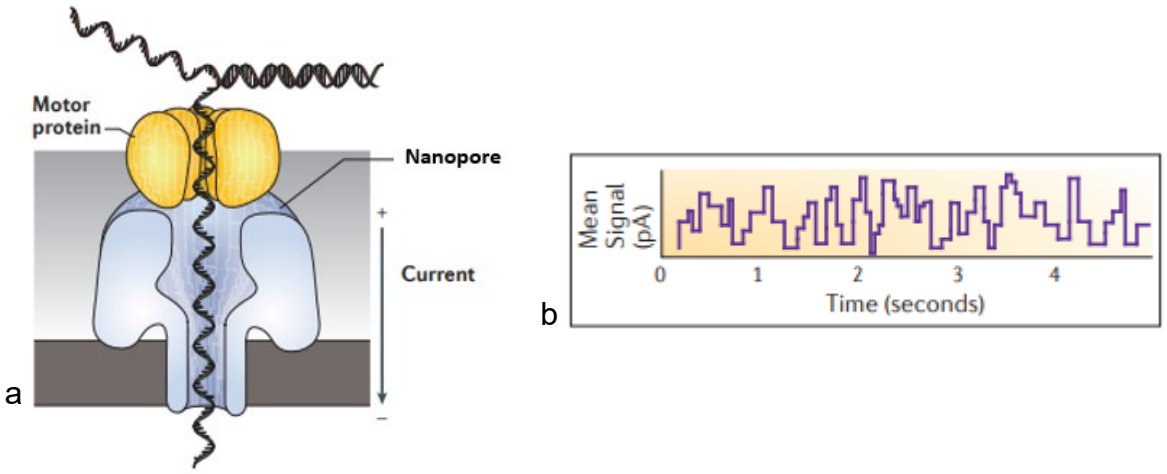


Figure 7. Schematic representation of ssDNA passing through the nanopore (A) resulting in an output reflecting the current shift caused by the ssDNA: squiggles (B) (figure adapted from Goodwin et al., 2016).

While high error rates have been observed upon the commercial release of nanopore sequencing technology in 2014-2015, immense research and development efforts in new pores, chemistry, basecalling algorithms and higher throughputs have enabled to increase the accuracy to a very high level. Single read accuracies of > 99.3% (Q20) will probably be reached in 2021 (ONT, 2021), therefore bringing the platform at the same accuracy levels next to Illumina and PacBio. When using Nanopore sequencing, obtained raw sequence data is further processed in a complete bioinformatics pipeline (including base calling, quality filtering, genome assembly, read alignments and polishing) to finally result in complete genomes.

Table 2. Comparison of the most popular next-generation sequencing platforms

	Second generation sequencing	*Third generation sequencing*	
	Illumina	**PacBio**	**Nanopore**
Sequencing method	Sequencing-by-synthesis	Single molecule	Single molecule
Polymerase	Free floating	Fixed to bottom	Absent
Read length	Short-read (150-250bp)	Long-read (~20kb)	Long-read (up to 200kb)
Real-time analysis	No	Yes	Yes
Turnaround time	24-72h	4-20h	2-48h
Raw read accuracy	> 99.5%	> 99.0%	> 99.3%
Initial cost	€€€	€€€	€
Portable model	No	No	Yes

One of ONTs devices, the MinION, is a portable sequencing platform, and has the potential to be applied directly in the field (Lamb et al., 2020). Until now, this has not been widely used in veterinary medicine, due to the remaining high cost, and as the raw read accuracy has been lower compared to Illumina (> 99%) (Rang et al., 2018). Therefore, the use of nanopore sequencing was limited to research and national surveillance settings, rather than applications in clinical diagnostics. However, MinION has already proven itself in the diagnosis of African Swine Fever, porcine viral enteric disease in piglets, and *P. multocida* in poultry (Costard et al., 2009; Theuns et al., 2018; Omaleki et al., 2020). In addition, McCabe et al. (2018) showed the rapid identification of viruses causing BRD using ONT from foetal lung cell cultures, and very recently (2021), nanopore sequencing was brought to the veterinary market as accessible tool for veterinarians for the identification of bacteria and viruses from BALf (PathoSense, Lier, Belgium). Unfortunately, standard protocols for the bioinformatics pipeline of nanopore sequencing are currently missing, and no information is available on the diagnostic accuracy of this method for the identification of *Mycoplasma* species from BALf.

Detection of (previous) *M. bovis* infection: antibody ELISA

Next to detecting the pathogen itself, antibodies can be detected in serum or milk. Robust detection of antibodies is possible with western blot analysis. Yet, this is a time consuming test. As a practical alternative, ELISA's are preferred in routine diagnostics (Andersson et al., 2019). Advantages of antibodies are that the animal does not need to be shedding the organism at the time of sampling and that longevity of antibody expression can last for 4-18 months (Petersen et al., 2018a; Vähänikkilä et al., 2019). Disadvantages are the lag between infection and seroconversion (2-3 weeks later), uncertainty of possible cross-reactivity with other *Mycoplasma* species, influence of animal age on antibody detection, difficulties in interpretation given the substantial variation between different ELISA's, and sometimes poor sensitivity (Parker et al., 2017; Petersen et al., 2018b; Parker et al., 2018). It is also concluded

that clinical disease in general is not correlated to the extent of the antibody response (Petersen et al., 2018a, Petersen et al., 2018b). The result is that most antibody ELISAs are not able to differentiate between healthy and diseased cows (Petersen et al., 2020). It is also possible that animals are shedding *M. bovis*, but antibodies cannot be detected in the bulk tank milk (Gille et al., 2018a). Whether this is a result of the lag time between infection and seroconversion, antibodies against other *M. bovis* associated diseases (*e.g.* pneumonia) without shedding of *M. bovis* in the milk, dilution of antibodies in the bulk tank milk by milk samples from *M. bovis* negative animals, or *M. bovis* binding to antibodies and interfering with the ELISA test is not clear.

An import remark is that the diagnostic test accuracy of these tests differs tremendously. Differences are shown between both commercially available and in-house tests, but results also depend on the target population, such as calves or adult cows, and healthy or diseased animals (Petersen et al., 2018b; Andersson et al., 2019; Petersen et al., 2020). Therefore, it is necessary to think about the aim of sampling either specific animals or the herd in general. For example, due to the delayed seroconversion, antibody ELISA might not be very useful in the detection of an acute outbreak and strategizing antimicrobial use. However, antibody ELISA could be useful in the context of animal purchase, especially in combination with culture or PCR testing. In that case both prior exposure and current infection will be covered. Nevertheless, there is still a chance that carrier animals, which are not shedding *M. bovis* at the time of purchase and did not develop antibodies, will be missed. The clearest benefit of antibody ELISA use is to diagnose at herd level, determining *M. bovis* positive and negative herds.

Recently, a point-of-care diagnostic method was developed, using a colloidal carbon test strip for the detection of *M. bovis* antibodies (Shi et al., 2020). Similar results were shown compared to antibody ELISA. Although cross-reactivity with more bovine *Mycoplasma* species should be investigated, this is a potentially interesting and rapid method for the identification of previous *M. bovis* contact.

Limitations of identification diagnostics

Even tough studies on individual samples show higher sensitivity of culture compared with qPCR assays (Castillo-Alcala et al., 2012; Parker et al., 2017; Wisselink et al., 2019), and different authors state that culture remains the ‘gold standard’ method for the detection of *M. bovis* (Parker et al., 2018; Calcutt et al., 2018), it seems not entirely correct to use this term ‘gold standard’. As made clear above, all methods have their advantages and disadvantages, and none of them obtains both sensitivity and specificity of 100% (Table 3).

Table. 3 Advantages and disadvantages of diagnostic methods identifying *M. bovis* from clinical samples (respiratory tract, milk, joint, body fluid, blood, ..)

	Culture	**PCR**	**Antibody ELISA**
Clinical sample	All	All	Blood, milk
Detection	Viable bacteria	DNA	Antibodies
TAT*	1-2 weeks	1-2 days	24 hours
Costs	€€	€€€	€
Advantages	• Evidence of live pathogen • Isolate available • Quantification possible • Detects multiple *Mycoplasma* species	• No viable *M. bovis* necessary • Rapid results • Pooling of samples possible • Quantification possible (qPCR)	• Shows previous challenge of *M. bovis* • Shedding of *M. bovis* not necessary
Disadvantages	• Viable *M. bovis* needed • Time consuming • Specific media needed • Unable to discriminate between *Mollicutes* without additional testing • Overgrowth of other bacteria possible • Shedding of *M. bovis* necessary	• No isolate obtained • Shedding of *M. bovis* necessary • Clinical interpretation sometimes difficult (non-viable *M. bovis*)	• No isolate obtained • Does not reflect present infection • Paired sera takes 3 weeks • Variable, but usually low sensitivity • Uncertainty of cross-reactivity with other *Mycoplasma* species

* Turnaround time: time between arrival in laboratory and test results, frequently depending on laboratory routines

The most important feature that a diagnostic test for *M. bovis* should hold, namely being rapid, is lacking for culture and ELISA. Therefore, whenever the only aim is to rapidly identify *M. bovis* in an acute outbreak, to determine control and prevention strategies or when antimicrobial therapy has already been started, PCR may be the best option. When interested in the *M. bovis* status of a herd, antibody ELISA can be the diagnostic method of choice. However, in both cases no isolate will be obtained, and further testing possibilities are limited. Nevertheless, in research and also in practice, for directing prevention and control measures, there is need for information concerning antimicrobial susceptibility and epidemiology of *M. bovis*. To get this information, obtaining an isolate is of great importance, and therefore culture is currently still needed. Therefore, focus should be on developing rapid culture-based identification methods. The current possibilities to identify and directly obtain an isolate of *M. bovis* in routine diagnostics are shown in Fig. 8.

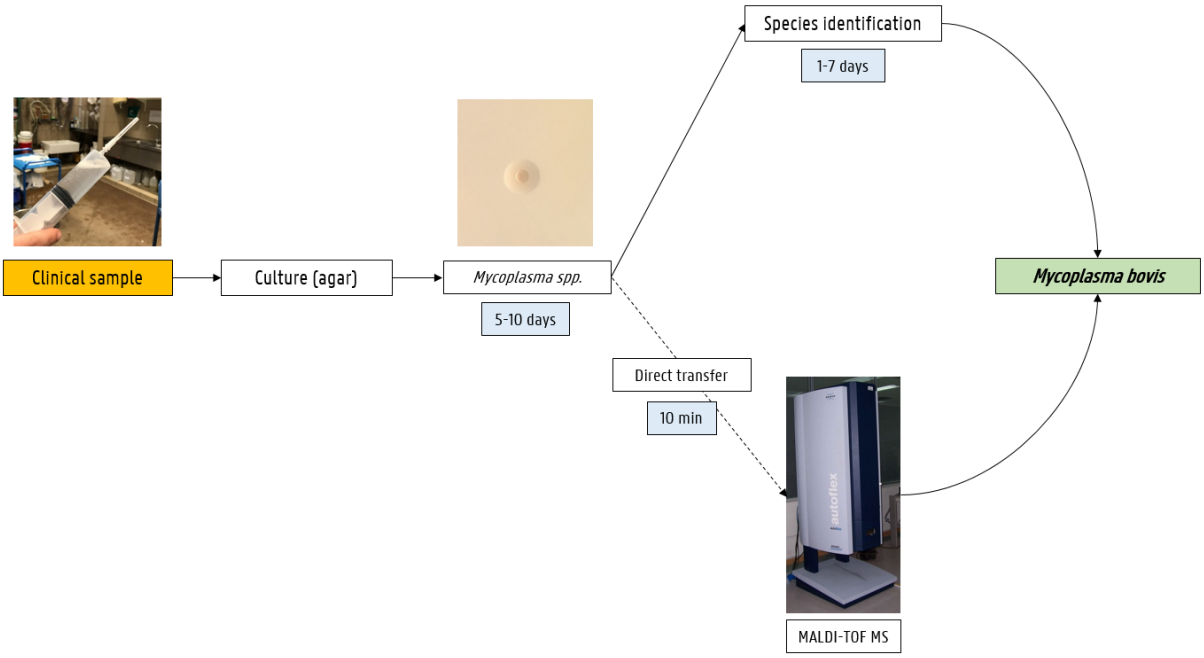


Figure 8. Current methods for the isolation of *M. bovis* with the advantage of the possibility to construct a databank of clinical isolates for additional antimicrobial susceptibility testing and epidemiologic studies, such as strain typing.

1.5 TYPING METHODS AND DIVERSITY OF *MYCOPLASMA BOVIS*

At the moment, strain typing of bacteria is almost strictly used in research settings. In routine diagnostics, bacterial identification remains mostly limited to (sub)species level (*e.g. Mycobacterium avium* subsp. *paratuberculosis*) or sometimes serotyping (*e.g. Salmonella* Dublin). Nevertheless, in the recent years the attention towards strain typing has increased, because of its potential to provide important epidemiological information in outbreaks or in legal cases for example. Currently, strain typing in routine diagnostics is mostly used in human medicine for tracing an outbreak back to its source, for example in methicillin-resistant *Staphylococcus aureus* (MRSA) and *Salmonella* outbreaks (Köser et al., 2012; Azarian et al., 2015; Hoffmann et al., 2016). Very recently, strain typing showed us the distribution of SARS-CoV-2 (COVID-19) over the world, and the importance of strain types with increased virulence or transmission capacities (Yang et al., 2020; WHO, 2020). Although the British variant of COVID-19 (SARS-CoV-2 VOC 2020 12/01) did not seem to show increased virulence at first, it immediate showed increased transmissibility (Public Health England, 2020; Davies et al., 2021). The fear for a more rapid spread and increased virulence, had great consequences on prevention measurements in many countries, such as the immediate closing of borders, and prolonged quarantine regulations.

Strain typing can also help in understanding the epidemiology of bacterial pathogens, such as *M. bovis,* and subsequently improve control and prevention measurements. For example, previously in this thesis it was mentioned that the question was raised whether *M. bovis* specific strains persisted in the veal calf sector, and whether these were more resistant against antimicrobials. Strain typing can help to answer these questions. In addition, when strain typing is performed on *M. bovis*, the isolate could be traced back to its source, and how and where *M. bovis* was transmitted might be clarified. Identifying virulence genes and antimicrobial resistance features of specific *M. bovis* strains might hold important information, and can help in more targeted control and prevention.

In the past, many molecular genetic tools for strain typing were explored to subtype *M. bovis* strains, but also phenotypical strain typing with MALDI-TOF MS has been studied (Becker et al., 2015). Also in strain typing diagnostics, whole genome based methods are gaining ground rapidly. When determining the strain typing method of use, it is important to keep the application in mind, but also whether cultivation is necessary to obtain high concentrations of

pure DNA or not. When including cultivation for *M. bovis*, significant increase in turnaround time and labor should be kept in mind.

Molecular genetic tools for strain typing

AFLP, RAPD, PFGE, and IS typing

Some previously used molecular methods for *M. bovis* strain typing are amplification fragment length polymorphism (AFLP) and random amplification of polymorphic DNA (RAPD), which are PCR based methods. Arbitrarily primed PCR, which has also been used, is a derivative of RAPD (Butler et al., 2001). Another often used method requiring an isolate, is pulsed-field gel electrophoresis (PFGE), a method based on separating DNA fragments by macrorestriction resulting in different band patterns per strain type (Kusiluka et al., 2000; Butler et al., 2001; McAuliffe et al., 2004; M. K. Soehnlen et al., 2011; Castillo-Alcala et al., 2012; Pinho et al., 2012). Previous methods were first compared by Kusiluka et al. (2000), who showed comparably robust and reproducible results of AFLP and PFGE on Danish *M. bovis* isolates. Later on, also McAuliffe et al. (2004) compared AFLP, RAPD, and PFGE on a subset of *M. bovis* isolates obtained in the United Kingdom. Although typing results of these methods are mostly in agreement, PFGE showed the least discriminatory power (McAuliffe et al., 2004). PFGE is also time consuming, more expensive, and results are difficult to compare between laboratories (Butler et al., 2001; McAuliffe et al., 2004; Castillo-Alcala et al., 2012; Menghwar et al., 2017). Even though good reproducibility of the RAPD results were shown by McAuliffe et al. (2004), this is usually a pitfall of the technique, and extra care should be taken in sample preparation (Butler et al., 2001; Sulyok et al., 2014). Differences in discriminatory power, reproducibility and ease of performance and interpretation of described methods are shown in Table 4.

Another molecular method which is used to type *M. bovis* is insertion sequence (IS) typing. This is a PCR based method, focusing on mobile genetic elements in the DNA of *M. bovis*. Southern blots are used to visualize the IS profiles (Miles et al., 2005; Thomas et al., 2005; Aebi et al., 2012; Gille et al., 2016). Although typing results of this method show a correlation with RAPD and AFLP results, there was no correlation observed with PFGE (Miles et al., 2005).

Multiple-locus variable-number tandem-repeat analysis (MLVA)

The above mentioned methods all have their applications, however discriminatory ability remains limited. Therefore, later on multiple-locus variable-number tandem-repeat analysis (MLVA) became more popular to compare strains from several geographical areas within countries (Amram et al., 2013; Spergser et al., 2013; Sulyok et al., 2014; Becker et al., 2015). This method is based on PCR and determines the variability between loci of repetitive DNA (Van Belkum, 2007) with the advantage of adjusting the number of loci studied in function of evolutionary pace (van Belkum, 2007; Pinho et al., 2012; Spergser et al., 2013). Also the discriminatory power can be increased with the number of loci analyzed (Becker et al., 2015). Sulyok et al. (2014) showed that MLVA was better able to distinguish closely related strains in Hungary, than multi-locus sequence typing (MLST). Unfortunately, comparison of MLVA results between and within laboratories remains difficult (Pinho et al., 2012; Register et al., 2015). Therefore, some authors have suggested that MLVA might best be used in combination with other techniques, such as MLST, RAPD or PFGE (Pinho et al., 2012; Sulyok et al., 2014). This would have its repercussions on costs, turnaround time and interpretation.

Single-locus sequence typing

When choosing for single-locus sequence typing, it is important to consider the locus of attention very carefully, as this will influence the discriminatory index enormously. In several countries, such as France and Spain, typing based on the *PolC* gene is frequently used (Becker et al., 2015; García-Galán et al., 2020), and appeared sufficiently discriminating to distinguish older and recent *M. bovis* strains from each other (Tardy et al., 2020). It was however not discriminatory enough to distinguish more recent Danish and French isolates (Becker et al., 2020; Tardy et al., 2020) and therefore probably not ideal for use of strain typing in diagnostics or for investigating recent outbreaks.

Multi-locus sequence typing (MLST)

Some of the above mentioned methods show either difficulties with both intra- and interlaboratory reproducibility and lack of discriminatory power (Table 4). A strain typing method that is characterized by both a good discriminatory power and high reproducibility is MLST. The principle of MLST is based on the DNA sequence of (parts of) seven housekeeping genes. A number is given to a specific allel sequence, and the combination of alleles are corresponding to one sequence type (ST) (Sabat et al., 2013). STs with similar

allelic profiles are grouped into clonal complexes (Rosales et al., 2015; Menghwar et al., 2017). MLST is a robust, and reproducible method to strain type *M. bovis*, and a public database (pubMLST) where all results can be collected is available for (inter)national comparison (Register et al., 2015; Rosales et al., 2015). Unfortunately, even though housekeeping genes are known to be poorly variable and were therefore expected to be constant through evolution, it was recently shown that part of the *M. bovis* population missed the *adh-1* locus, one of the genes that were used in the *M. bovis* MLST scheme. This observation led to the need for an adjusted MLST scheme, which was proposed by Register et al. (2020), and implemented immediately (PubMLST, *M. bovis*).

Although MLST is a solid method to compare *M. bovis* isolates over countries, it is not ideal. The discriminatory power is sometimes insufficient (Sulyok et al., 2014; Becker et al., 2015), and the risk of losing another housekeeping gene by evolution in the future is imaginable. For better comparison between countries, discriminatory power, and reproducibility, WGS can be the solution (Tardy et al., 2020).

Next-generation sequencing methods

As mentioned in the previous chapter, next-generation sequencing (NGS) becomes more attractive as an all-in-one diagnostic tool. With the reduced cost, this also becomes more available for strain typing. Considering its high reproducibility and providing genetic information on the entire genome, NGS could be a great opportunity for surveillance and disease outbreaks. In addition, NGS may even be superior to the more classical methods, such as PFGE and MLST, as shown in human medicine (Leekitcharoenphon et al., 2014; Deng et al., 2016).

When whole genomes of a pathogen are obtained and compared to each other, one can choose to approach the genome on different levels: (1) focusing on specific genes such as species-specific MLST genes, (2) the core genome (cg) which is similar between genomes of the isolates from the same species or (3) the whole genome (wg), which is composed of the core genome and every strains accessory genome (Fig. 7). When multiple whole genomes of one species are assembled, this is called the pan genome. To illustrate this, when performing cgMLST, not only the alleles of seven house-keeping or MLST genes are included, but alleles of multiple genes located on the core genome are used. By performing wgMLST, also some accessory loci are included (Maiden et al., 2013). To compare genomes on the level of

nucleotides, instead of loci or genes, single nucleotide polymorphism (SNP) analysis can be performed. The same distinction can be made for SNPs present in the core genome (cgSNP) or whole genome (wgSNP). The common principles of cg and wg sequencing, are visualized in Fig 9.

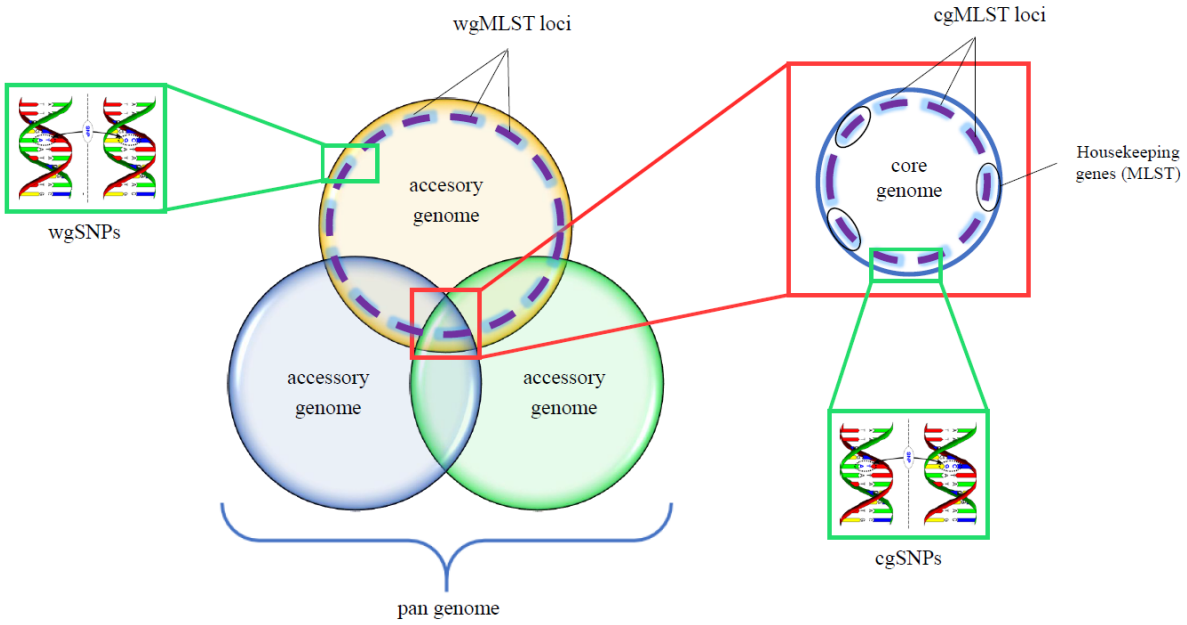


Figure 9. Visualization of the core and accessory genomes from different bacterial strains, and the possible strain typing methods (MLST, cgMLST, wgMLST, cgSNP, wgSNP) (source SNPs figure: dnabaser.com)

Kinnear et al. (2021) compared MLST, core genome MLST (cgMLST), core genome single nucleotide variant (cgSNP) and whole genome nucleotide variant (wgSNP) analysis on 129 *M. bovis* isolates. Although all methods provided highly comparable results, MLST, which only compared 7 house-keeping genes had the lowest discriminatory power. The highest resolution was obtained with wgSNP (Kinnear et al., 2021). In contrast, another study showed lower discriminatory power of wgSNP compared to cgMLST (Tardy et al., 2020). Beside technical differences (bioinformatics pipelines are often not standardized), the differences in discriminatory power between studies can be explained by the divergence within the studied populations. When more variety in genomes is observed, the core genome becomes relatively smaller and the accessory genome larger. Also overall genome quality and completeness are very important, as when large parts of the genomes are missing, these parts cannot be included in the core genome analysis. This is especially the case for the reference genome, when included in the SNP analysis (Besser et al., 2018). Long-read sequencing methods (*e.g.* SMRT or nanopore sequencing) can possibly help increase the discriminatory power, as less

gaps in the genome are observed compared to short-read sequencing methods (*e.g.* Illumina) (Fig. 10). On the contrary, the lower read accuracy of long-read sequencing methods should be kept in mind (Rang et al., 2018). Therefore, short reads can help improve the quality of base-reading in each individual position by increasing the coverage. Combining both long-read sequencing for complete genomes with Illumina reads ('hybrid-assembly') can then also improve genome assemblies (Goldstein et al., 2019). However, due to the delay in results, this is not recommended for routine diagnostic purposes.

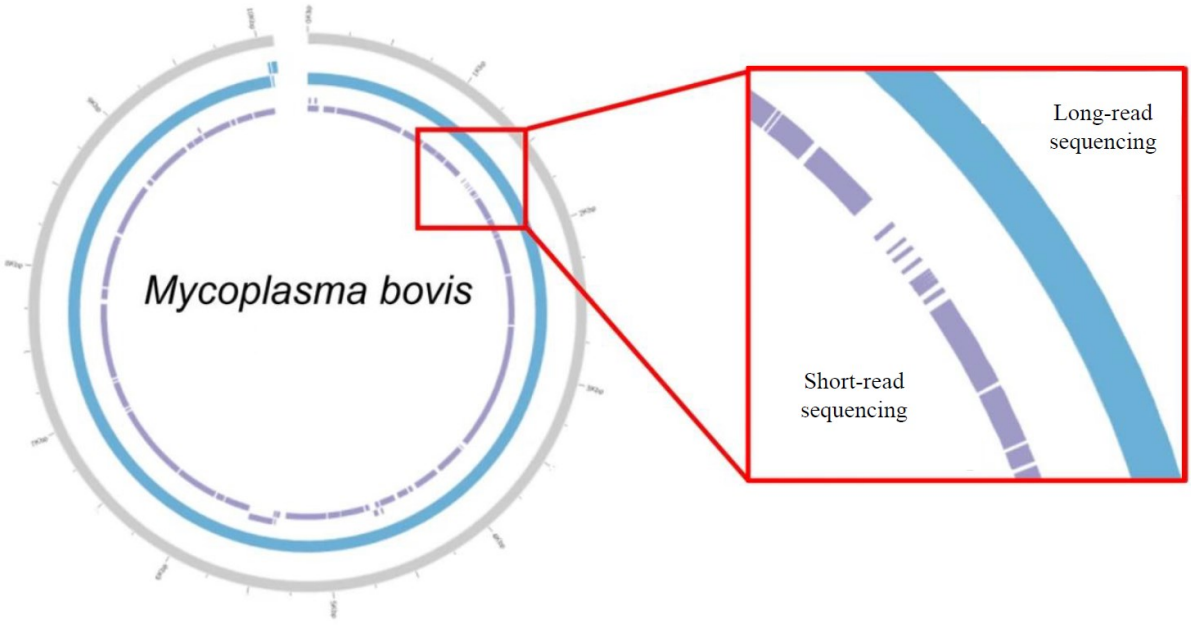


Figure 10. Short-read sequencing results in multiple short contigs and a genome with gaps (purple), whereas with long-read sequencing mostly full genomes are covered for *M. bovis* (blue) in comparison to the reference genome (grey) (figure adapted from Vereecke et al., 2020).

Apart from the sequencing method that is used, optimizing the bioinformatics pipelines is very important to obtain reliable high quality and complete genomes. This might be more of a technical challenge and a disadvantage of NGS compared to MLST as sequencing method (Register et al., 2020; Kinnear et al., 2021). Nevertheless, these difficulties did not discourage researchers, as NGS for strain typing *M. bovis* and obtaining epidemiological information on *M. bovis* strains from different countries, has been widely explored in recent years (Parker et al., 2016; Yair et al., 2020; Tardy et al., 2020; Kumar et al., 2020; Kinnear et al., 2021). In these studies, short-read sequencing by Illumina was used. However, as previously mentioned, where Illumina is highly time-consuming and requires a significant upfront investment, long-read nanopore sequencing is known to create faster results and is a highly accessible tool for routine diagnostics and surveillance due to its portable form (Theuns et al.,

2018). Unfortunately, to date their use is limited to research or national surveillance settings, and it has not been integrated in bacterial routine diagnostics.

Phenotypic methods

MALDI-TOF MS, a phenotypic method, has also been described as a strain typing method using French isolates. The protein profiles of *M. bovis* obtained by MALDI-TOF MS showed the same topology as MLVA and MLST. However MALDI-TOF MS and MLVA showed a higher discriminatory power of individual isolates than MLST, and reflected the loss of heterogeneity in the French *M. bovis* population (Becker et al., 2015). MALDI-TOF MS seems like a promising tool for phenotypic strain typing of *M. bovis,* but so far no other studies showed the use of MALDI-TOF MS as strain typing method. Therefore, it remains unclear whether this is a reproducible method, can be compared between laboratories and if its applicable to all *M. bovis* populations. Most likely, a standardized protocol with extra attention to growth phase and culture conditions is necessary (Sauer et al., 2008; Sloan et al., 2017).

Table. 4 Overview of different strain typing methods for *M. bovis* showing characteristics, advantages and disadvantages

Method	Discriminatory power	Reproducibility (interrun/laboratory)	Ease of performance	Ease of interpretation
AFLP	++	+/-	-	-
RAPD	+	-	++	+/-
PFGE	+	+/-	+/-	+
MALDI-TOF	+	ND	+	+/-
IS typing	+	+-	+	+/-
PolC typing	+-	++	+	++
MLVA	+++	--	+	++
MLST	++	++	+	++
NGS	+++	++	+/-	+/-

AFLP: amplification fragment length polymorphism; RAPD: random amplification of polymorphic DNA; PFGE: pulsed-field gel electrophoresis; MALDI-TOF: matrix-assisted laser desorption ionization-time of flight mass spectrometry; IS typing: insertion sequence typing; MLVA: multiple-locus variable-number tandem-repeat analysis; MLST: Multi-locus sequence typing; NGS: next-generation sequencing (Illumina, PacBio, ONT); ND: not determined. Interpretation of very good (+++), good (++), acceptable (+), dubious (+-) or bad (-) are based on a combination of the authors experience, specific *M. bovis* literature and Sabat et al., 2013.

Molecular diversity of *M. bovis*

There are many studies, showing the widespread heterogeneity of *M. bovis* strains all over the world. For example, in Japan already 52 different sequence types (STs) have been identified, while in western Canada, 30 STs have been described (Hata et al., 2019; Kinnear et al., 2021). Some of the Canadian STs were also identified on other continents, such as ST52 (Europe,

Asia and Oceania), and ST21 (Europe and Asia) (Kinnear et al., 2021). Not surprisingly, due to the bilateral trade between both countries, isolates from Canada and the USA clustered together as well (Kumar et al., 2020; Kinnear et al., 2021). Depending on the subgroup, Japanese STs clustered together with American, European, Australian, Israeli and/or Chinese isolates, suggesting exchange of *M. bovis* isolates between these regions (Hata et al., 2019). Chinese isolates cluster together with isolates from the USA, Australia, and Israel (Liu et al., 2020), which is in line with other studies separating most European isolates apart from Australian, Chinese and American isolates (Kumar et al., 2020; Yair et al., 2020). Nevertheless, some of the American isolates seem to cluster together with European isolates, such as the Swiss and Lithuanian isolates (Kumar et al., 2020). Israel purchased animals and probably imported *M. bovis* from Europe, China and Australia, which is also reflected in the way Israelian isolates cluster with STs described in these countries (Yair et al., 2020). It is however observed that Australian isolates show notable genetic similarities, while isolates from the USA and Europe show much more divergence (Parker et al., 2016; Kumar et al., 2020; Register et al., 2020; Yair et al., 2020). This might be due to strict biosecurity practices in Australia, such as quarantine rules for the imported livestock, which possibly helped to prevent the introduction of multiple strains over time into the country (Parker et al., 2016).

In Europe and Japan, a shift in homogeneity was observed in the *M. bovis* population (Becker et al., 2015; Bürki et al., 2016; Hata et al., 2019; Tardy et al., 2020). First in Denmark, more genetic variation was observed in isolates from 1992 on with AFLP (Kusiluka et al., 2000), while a more recent publication identified again homogeneity within isolates obtained after 2000 with cgMLST and wgSNP (Tardy et al., 2020). Such a shift was also observed in France with MLST, and MLVA, around 2000 (Becker et al., 2015), and with PFGE in 2003 (Arcangioli et al., 2012), suggesting clonal emergence of *M. bovis* in these countries (Becker et al., 2015; Tardy et al., 2020). Although in the Scandinavian countries (Denmark, Sweden, Finland) a certain level of homogeneity was observed with wgSNP and cgMLST (Haapala et al., 2018; Tardy et al., 2020), more heterogeneity compared to the Scandinavian countries was observed between isolates from the Netherlands and France after 2000 (Tardy et al., 2020). This is in line with earlier reports from France based on *PolC* typing, where st2 and st3 were the only abundantly present strain types in France, but recently also st5 was isolated for the first time (Becker et al., 2015; Becker et al. 2020). Also in Hungary and the United Kingdom, high heterogeneity of isolates has been observed (McAuliffe et al., 2004; Sulyok et al., 2014). One possible reason could be the intensive (inter)national cattle movements in these countries

with relatively more heterogeneity (McAuliffe et al., 2004; Sulyok et al., 2014; Tardy et al., 2020).

Strain types and disease course

The question whether certain strain or sequence types can be related to different *M. bovis* associated diseases or severity of clinical signs was raised many times. For example, some countries seem to have more problems with *M. bovis* mastitis (*e.g.* Israel, Switzerland), where others face more respiratory diseases caused by *M. bovis* (*e.g.* France). Both in Switzerland and Israel, a sudden increase in mastitis cases was observed. Therefore, introduction of a new or mutated *M. bovis* strain with another predilection site preference was suspected (Bürki et al., 2016; Lysnyansky et al., 2016). Although ST10 has been isolated frequently from these clinical mastitis cases in Israel and China (Lysnyansky et al., 2016; Liu et al., 2020), ST10 was also frequently isolated from the respiratory tract and joints of Chinese calves (Menghwar et al., 2017; Guo et al., 2020). So far, many studies were unsuccessful in identifying an association between strain type and anatomical location, no matter what strain typing method was used (Kusiluka et al., 2000; Biddle et al., 2005, Rosales et al., 2015; Parker et al., 2016; Garciá-Galàn et al., 2020; Tardy et al., 2020).

During *M. bovis* outbreaks, different strain types can be identified from the same animal or in the same herd (Soehnlen et al., 2011; Sulyok et al., 2014; Rosales et al., 2015; Garciá-Galàn et al., 2020; Becker et al., 2020). However, whether different *M. bovis* strains are circulating probably depends on the management style, being an open or closed herd (Butler et al., 2001), as several studies show one dominant type in an outbreak (Butler et al., 2001; Aebi et al., 2012; Arcangioli et al., 2012; Becker et al., 2020). In feedlots, clonal spread of a single *M. bovis* strain has been demonstrated mainly in young calves. Later on in the production cycle, multiple genotypes are generally isolated (Castillo-Alcala et al., 2012; Timsit et al., 2012; Becker et al., 2020). In an Australian dairy herd however, the same *M. bovis* strain was found for seven consecutive years (Parker et al., 2016). Also in the Alpine areas strain typing analysis with RAPD and MLVA suggested the re-emergence of the same strain type in 2009 and outbreaks in 2010-2011, after the first outbreak in 2007 (Spergser et al., 2013).

No direct association between *M. bovis* strain type and health status has been described up till today (Parker et al., 2016; Register et al., 2019; Garciá-Galàn et al., 2020; Yair et al., 2020), although it was shown that isolates from dead animals showed more antimicrobial resistance

than those obtained from healthy animals (Jelinski et al., 2020). Whether this is associated with strain type or antimicrobial therapy is not clear. In China and Japan, certain STs were associated with increased antimicrobial resistance (AMR) (Hata et al., 2019; Liu et al., 2020), and it has been shown that st3 isolates (*PolC* typing) acquire resistance against fluoroquinolones more easily than st2 isolates under antimicrobial selection pressure. These associations can have an influence on the efficacy of the therapy and therefore indirectly on health status (Khalil et al., 2015; García-Galán et al., 2020).

1.6 ANTIMICROBIAL SUSCEPTIBILITY OF *MYCOPLASMA BOVIS*

As described earlier in this thesis, *M. bovis* is inherently resistant against beta-lactam antibiotics and (potentiated) sulphonamides. It is however more worrisome how easy *M. bovis* can acquire resistance in the presence of an antimicrobial selection pressure (Sulyok et al., 2017). This can at least partly be explained by the small genome and lack of certain genetic information coding for DNA repair mechanisms, resulting in a high mutation rate in this species (Rocha et al., 2005). In the last two decades, many studies explored antimicrobial susceptibility of *M. bovis* in different parts of the world. Most of the research is based on phenotypic antimicrobial susceptibility testing (AST), but an increase in the determination of antimicrobial susceptibility based on molecular genetic methods is seen in the last few years (Sulyok et al., 2018; Hata et al., 2019; Kinnear et al., 2020).

Phenotypic antimicrobial susceptibility testing

Conventional antimicrobial susceptibility testing and pitfalls

Performing and interpreting phenotypic AST of *M. bovis* knows many difficulties and pitfalls. These can roughly be subdivided into long turnaround time, absence of standard protocols, and interpretation of results.

First of all, the long turnaround time of approximately 2 weeks, often prevents the use of AST for *M. bovis* in routine diagnostics (Ben Shabat et al., 2010; Sulyok et al., 2018). Secondly, comparing the results of different phenotypic AST studies is often difficult, as there are no internationally recognized standardized methods for AST of *M. bovis* and validated reference values for quality control strains aren't available either (Gautier-Bouchardon, 2018). Usually, the minimum inhibitory concentration (MIC) is determined in phenotypic AST methods. The MIC is the lowest concentration of an antimicrobial that will inhibit visible growth or

metabolism after its optimal incubation period *in vitro* (Hannan, 2000). To determine the MIC for *M. bovis*, some studies use the agar dilution assay (Uemura et al., 2010; Siugzdaite et al., 2012; Sato et al., 2013, 2017; Gautier-Bouchardon et al., 2014; Kong et al., 2016; Khalil et al., 2017; Hata et al., 2019; Becker et al., 2020; Tardy et al., 2020), the gradient strip test (Francoz et al., 2005; Gerchman et al., 2009) or flow cytometry (Soehnlen et al., 2011). However, broth microdilution (BMD) is considered the 'gold standard' method for most bacterial species (Lallemand et al., 2016), and in the last few years, an increasing number of studies are reporting the use of BMD as exemplified in Table 5.

Nevertheless, even in these studies no standardized protocol is followed, as different broth compositions, inoculum concentrations, pH indicators and incubation periods are described, possibly affecting the results and preventing straightforward one on one comparison between these studies (Table 3) (Thomas et al., 2003; Lallemand et al., 2016).

The last pitfall, is the subsequent interpretation of obtained MIC values for *M. bovis*, as there are no clinical breakpoints available translating *in vitro* results into clinical expectations. Even though this is probably questionable, several studies interpret *M. bovis* MIC values with the clinical breakpoints provided by CLSI standards for other bovine respiratory pathogens or human *Mycoplasma* species (Heuvelink et al., 2016; Anholt et al., 2017; Becker et al., 2020; Tardy et al., 2020) to categorize isolates as 'resistant', 'intermediate' or 'susceptible' to an antimicrobial agent (Table 3). In addition, when choosing antimicrobial therapy based on antimicrobial susceptibility results, care should be taken with the interpretation of the 'intermediate' result. Intermediate results could reflect three different principles, being (1) the uncertain effect of the antimicrobial drug used on therapy outcome (2) increased antimicrobial concentration in certain body parts may still result in therapy success or (3) as a buffer zone for technical variation. To clarify the meaning of the category 'intermediate', the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recently adjusted this term to 'susceptible, increased exposure' (EUCAST), while the Clinical and Laboratory Standards Institute (CLSI) added the category 'susceptible, dose dependent', and the addition of '^' to the 'intermediate' category indicating the possible accumulation of the antimicrobial agent in certain body parts, and therefore possible therapy success in those circumstances (Kahlmeter et al., 2019). The full definitions of 'intermediate' and 'susceptible, increased exposure' are shown in Table 6.

Table 5. Overview of antimicrobial susceptibility publications of *M. bovis* over 2015-2020.

Study	N	Herds	Year	Country	Sector	Health status	MIC Method	Start concentration	Incubation time	Interpretation Method
A	73	55	2011-2014	BEL, DEU, ITA	Dairy cattle	NA	BMD	10^4 UCC/ml	18h – growth*	Mode; MIC_{50}-MIC_{90}
B	95	NA	2008-2014	NLD	Veal & dairy	Diseased or dead	BMD	0.5 x 10^3-10^5 CCU/ml	48h	MIC_{50}-$MIC_{90;}$ CLSI
C	32	32	2011-2013	CHN	Feedlot	Symptomatic	BMD	10^3-10^5 CCU/ml	NA	CLSI
D	40	NA	2012-2014	CAN	Bison	Dead	BMD	2 x 10^3-10^5 CUU/ml	96h	MIC_{50}-$MIC_{90;}$ CLSI
E	226	NA	2014-2015	CAN	Feedlot	Diseased	BMD	0.5 McFarland	24-96h	$MIC_{50;}$ CLSI
F	58	51	2010-2011	JPN	Dairy calves	Asymptomatic	ADM	10^4-10^5 CFU/ml	72h	NA
G	26	NA	2000-2014	FRA	NA	Diseased	ADM	10^4-10^5 CFU/ml	5d	CLSI; Hannan, 2000
H	156	156	2010-2012	FRA, HUN, ESP, GBR	NA	Diseased	BMD	5 x 10^5 CFU/ml	24-48h	MIC_{50}-MIC_{90}
I	210	NA	1978-2009	CAN	Beef, dairy, veal	Diseased	BMD	10^3-10^5 CFU/ml	24-96h	MIC_{50}
J	232	224	2014-2016	FRA, HUN, ITA, ESP, GBR	NA	Diseased	BMD	5 x 10^5 CFU/ml	24-48h	MIC_{50}-MIC_{90}
K	203	52	1993-2018	JPN	Unknown	Unknown	ADM	10^3-10^5 CFU/ml	NA	MIC_{50}-MIC_{90}
L	211	31	2006-2018	CAN, USA	Feedlot	All	BMD	10^3-10^5 CFU/ml	48-72h	CLSI
M	39	14	2016-2018	FRA	Veal	Unknown	ADM	10^4-10^5 CFU/ml	5d	CLSI; MIC_{90}
N	81	NA	1981-2017	DNK, SWE, FRA, NLD, EST, FIN	NA	NA	ADM	10^4-10^5 CFU/ml	5d	CLSI
O	50	23	2018-2019	CHN	Dairy	Diseased	BMD	10^3-10^5 CCU/ml	48h	MIC_{50}-MIC_{90}
P	95	22	2016-2019	ESP	Beef, dairy	Healthy/diseased	BMD	10^3-10^5 CCU/ml	48h	MIC_{50}-MIC_{90}

A: Barberio et al., 2016; B: Heuvelink et al. 2016; C: Kong et al., 2016; D: Suleman et al., 2016; E: Anholt et al., 2017; F: Sato et al., 2017; G: Khalil et al., 2017; H: Klein et al., 2017; I: Cai et al., 2018; J: Klein et al., 2019; K: Hata et al., 2019; L: Jelinksi et al., 2020; M: Becker et al., 2020; N: Tardy et al., 2020; O: Liu et al., 2020: P: Garciá-Galan et al., 2020. Country name is coded following ISO 3166-1: alpha-3 code; *: positive growth control. MIC = minimum inhibitory concentration, NA = not available; BMD = broth microdilution, ADM = agar dilution method, UCC = unit changing color; CCU = colour changing untis, CFU = colony forming units, CLSI = clinical breakpoints following CLSI guidelines, MIC_{50}-MIC_{90} = the lowest MIC at which at least 50% or 90% of the isolates were inhibited in their growth

Table 6. Recent changes in the definitions of 'intermediate' isolates by CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) as cited by Kahlmeter et al. (2019)

Intermediate (I)	CLSI	A category defined by a breakpoint that includes isolates for which MICs or zone diameters are within the intermediate range; the drug approaches usually-attainable blood and tissue levels, and response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated. The I category also includes a buffer zone for inherent variability in test methods, which should prevent small, uncontrolled technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.
Intermediate ^	CLSI	An I with a "^" in document M100 Table 2 is used to describe agents that have the potential to concentrate at an anatomical site.
Previous definition of 'intermediate'	EUCAST	A microorganism is defined as intermediate by a level of antimicrobial agent activity associated with uncertain therapeutic effect. It implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of the drug can be used; it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.
Current definition of 'susceptible, increased exposure'	EUCAST	A microorganism is categorized as "susceptible, increased exposure" when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection.

Another way of interpreting MIC results is distinguishing the wild type (WT) population from strains with acquired antimicrobial resistance, also called the non-wild type population (non-WT) by means of the epidemiological cut-off (ECOFF) value or wild type cut-off. The ECOFF is independent of evolutionary changes, and is therefore believed to remain the same for a particular organism and antimicrobial agent throughout time, independent of geographic location or host species (Silly, 2012). Unfortunately, the epidemiological criterium is not designed to predict *in vivo* effectiveness of the antimicrobial agent, but it can be used as a sensitive threshold to predict antimicrobial resistance emergence in specific bacterial populations, including *M. bovis*. Therefore, the ECOFF is most useful in monitoring and surveillance of AMR development (Turnidge et al., 2006; Silley, 2012).

The methodology on how the ECOFF should be determined is currently not harmonized (Silly, 2012). The ECOFF can be determined using visual estimation ("eyeball") or statistical methods (*e.g.* normalized resistance interpretation (NRI) or the iterative statistical method (ISM)) (Turnidge et al., 2006; Kronvall, 2010; Callens et al., 2016). The visual estimation method distinguishes two populations of bacterial strains based on a bimodal MIC distribution in those with (non-WT) and without (WT) acquired resistance (Turnidge and Paterson, 2007; Toutain et al., 2017). The statistical methods are based on the assumption that the wild type

population is symmetrical round its midpoint. Data are either normalized (NRI) or log-transformed (ISM), and based on mean and standard deviations, the ECOFF is determined (Turnidge et al., 2006; Kronvall and Smith, 2016).

Foremost, *M. bovis* susceptibility results were reported as MIC_{50} and MIC_{90} values as shown in Table 3, being the lowest MIC at which at least 50% or 90% of the isolates were inhibited in their growth. The MIC_{50} is expected to show the potency of an antimicrobial against a bacterial species, whereas the MIC_{90} indicates potential and first stages of specific bacteria developing resistance (Lysnyansky and Ayling, 2016). However, this assumes that the MIC_{50} and MIC_{90} are always situated in the WT or non-WT population, respectively. Unfortunately, these indicators are subject to the tested population, do not hold a lot of clinically relevant information and are difficult to interpret related to acquired resistance when MIC distributions are not presented as well (Turnidge et al., 2006; Schwarz et al., 2010). Nowadays, most *M. bovis* populations have shifted to increased acquired resistance, showing limited significance of the MIC_{50} and MIC_{90} as parameters for antimicrobial susceptibility.

Whenever one is using the ECOFF, clinical breakpoints or MIC_{50}/MIC_{90} for the interpretation of AST results, one should be very cautious in the conclusions that are drawn. To support this, an example of the different outcomes of ECOFF (visual estimation method), clinical breakpoints for bovine respiratory pathogens, and MIC_{50}/MIC_{90} for a hypothetical antimicrobial distribution of MIC values of *M. bovis* isolates is shown in Fig. 11.

In conclusion, many variables can influence the results and interpretation of AST in *M. bovis*. Therefore there is a clear need for standardization of AST protocols and *M. bovis* specific clinical breakpoints, to be able to compare AST results and draw straightforward conclusions, both epidemiologically and clinically.

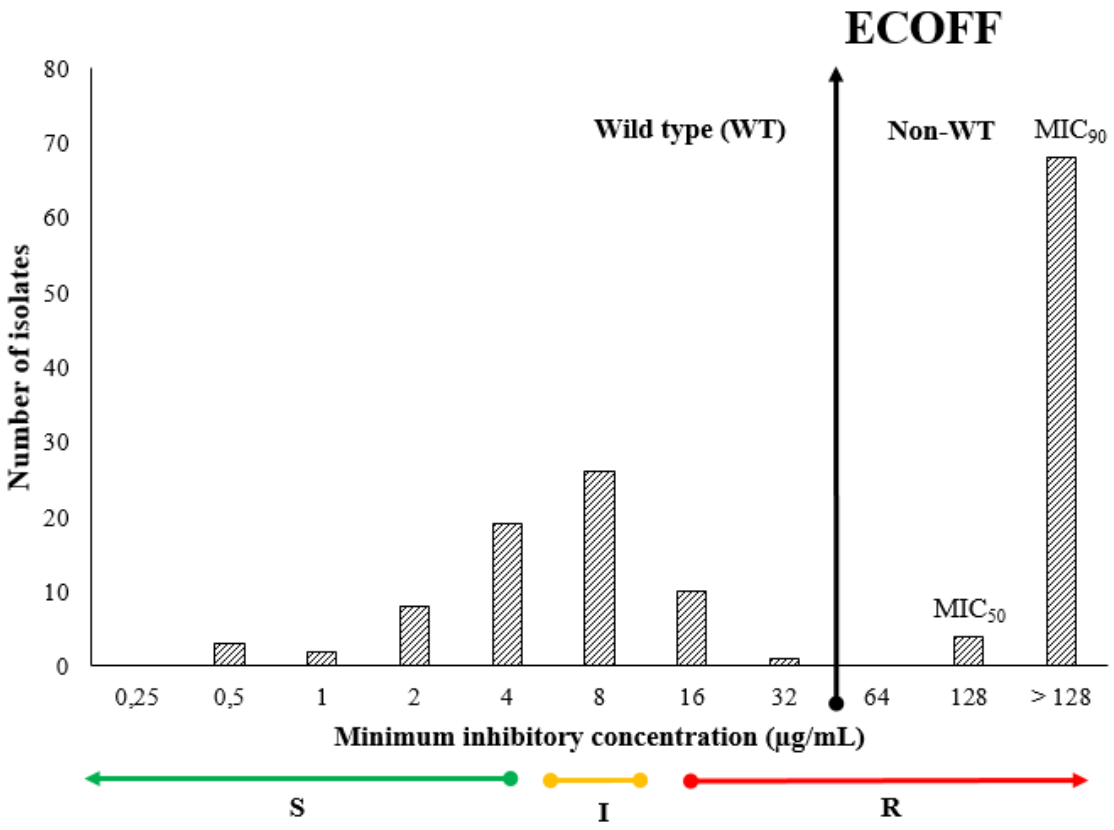


Figure 11. Hypothetical bimodal distribution of *M. bovis* MIC-values for an antimicrobial showing the determination of the epidemiological cut-off (ECOFF) by the visual estimation dividing the population in wild type (WT) and non-wild type (non-WT) isolates. The corresponding interpretation of the clinical breakpoints for bovine respiratory pathogens (CLSI, VET01-S3, 2015) is included, categorizing the isolates in either susceptible (S $\leq$ 4 µg/mL), intermediate (I = 8 µg/mL), or resistant (R $\geq$ 16 µg/mL), and showing a substantial difference between clinical and epidemiological interpretation. Also MIC_{50} (128 µg/mL) and MIC_{90} (> 128 µg/mL) were calculated. Although a large dataset was used, both values might be reflecting the non-WT population instead of WT and non-WT for MIC_{50} and MIC_{90}, respectively.

Antimicrobial susceptibility testing with MALDI- TOF MS

By the introduction of MALDI-TOF MS into microbiological laboratories, not only bacterial identification possibilities of this technique have been explored, but also methods to identify AMR. Two methods that are commercially available, are the MBT STAR®-BL and MBT STAR®-CEPHA. These methods detect a mass shift when beta-lactamase activity hydrolyzes the beta-lactam antibiotic (Bruker Daltonics). These methods are not of value for *M. bovis,* due to its inherent resistance against beta-lactam antibiotics. However, antimicrobial

resistance determination can also be based on the detection of specific peaks in mass spectra, such as for MRSA, or be based on the ability to grow within the presence of an antimicrobial agent (MBT-ASTRA) (Josten et al., 2013; Lange et al., 2014). The latter, showed to be applicable to many bacteria and antimicrobials, both in human medicine and veterinary medicine (Lange et al., 2014; Ceyssens et al., 2017; Van Driessche et al., 2018), resulting in the determination of antimicrobial resistance profiles within 3 hours after obtaining pure cultures (Sparbier et al., 2016; Van Driessche et al., 2018). The advantage of this phenotypic technique is that knowledge of underlying resistance mechanisms is not necessary, as relative growth between the isolate with and without the antimicrobial is determined. Unfortunately, to be able to standardize this method for *M. bovis*, cultivation of an isolate is necessary (additional 1-2 days), the start concentration should be easily determinable (which for *M. bovis* usually takes 'backwards counting') and clinical breakpoints are necessary to categorize isolates as either resistant or susceptible for final cut-off values. Therefore, this method seems inappropriate or at least far-fetched for AST of *Mycoplasma* species, at least until clinical breakpoints are available.

Molecular genetic tools for antimicrobial susceptibility testing

In the quest to find other more rapid and standardized methods for the determination of AMR in *M. bovis*, the popularity of molecular genetic methods seems rising (Sulyok et al., 2018; Hata et al., 2019; Kinnear et al., 2020). Hereunder some of these techniques will be explained.

qPCR based methods

Beside the use of qPCR as identification method for *M. bovis* (see 'diagnostics'), it is also possible to detect antimicrobial resistance genes and specific mutations with qPCR (Maurin, 2012). Target DNA will be amplified and simultaneously fluorescence production will be detected, which makes this method more rapid than conventional PCR (Maurin, 2012). In human medicine, qPCR is already widely implemented in the rapid detection of antimicrobial resistance genes, such as for MRSA and vancomycin resistance in *Enterococcus* species, and detection of mutations, such as in *Mycoplasma pneumoniae* (Peuchant et al., 2009; Bourdon et al., 2010; Patel et al., 2011). Also several qPCR assays are commercially available for the detection of macrolide resistance by point mutations in *Mycoplasma genitalium*, which is a sexually transmitted organism in humans. The diagnostic accuracy of three of these kits were explored, showing high diagnostic accuracy (sensitivity 95-100%, specificity 95-97%) (Le

Roy et al., 2020). Unfortunately, such assays are not (yet) commercially available for *M. bovis*.

In 2009, Ben Shabat et al. explored a qPCR method for the differentiation between fluoroquinolone resistant and susceptible isolates. Unfortunately, this method was only targeting one mutation in the *parC* quinolone resistance-determining region (QRDR). However, most *M. bovis* isolates are only resistant against fluoroquinolones when an additional mutation in the *gyrA* gene is present as well, although mutations in the *gyrA* gene alone can also increase MIC values (Lysnyansky et al., 2009). More recently, real-time PCR based assays, such as melt curve analysis of mismatch amplification mutation assays (Melt-MAMA) and high resolution melting (HRM) assays, were explored by Sulyok et al. (2018) to discriminate between *M. bovis* isolates with 'low' and 'high' MIC value from lung and nasal swabs. Within one day, results are available from clinical samples for tetracyclines, macrolides, lincosamides and fluoroquinolones, while for spectinomycin an initial isolate should be obtained before DNA extraction for the MAMA assay, which takes about 3-4 days (Sulyok et al., 2018). The latter is necessary because the HRM assay is not able to simultaneously detect mutation T1199C (associated with tetracycline resistance) and C1192A (associated with spectinomycin resistance), and the MAMA assay directly on the clinical sample showed a high false result rate (53.3%) for spectinomycin, due to a lower sensitivity of the assay (10^5 copies/reaction compared to 10^2-10^3 for all other antimicrobials). Unfortunately, only discrimination between low and high MIC values were possible, which can be problematic for the clinical interpretation when isolates with 'moderate' MIC values are tested, as no clinical breakpoints are available. Melting curve analysis using hybridization probes on milk samples, also detects mutations in the target area of the genome within 3 hours for several antimicrobials (Hata et al., 2019). However, it was still recommended to perform DNA sequencing afterwards for better interpretation, as this methods also identifies silent mutations (Hata et al., 2019).

Next-generation sequencing methods

More recently also NGS has come to the forefront in the determination of AMR in veterinary medicine (Owen et al., 2017; Stanford et al., 2019). However, where most pathogens contain well known AMR genes (*e.g. Pasteurellaceae*) (Owen et al., 2017), the challenge for *Mycoplasma* spp. is that resistance is mainly determined by point mutations (Table 7). To determine single nucleotide mutation throughout the entire genome, obtaining high quality

genomes and high accuracy of the used method are very important. Missing or altered nucleotides could easily result in false non-synonymous mutations (nsSNPs), and therefore mistakenly be (dis)associated with AMR phenotype. Those nsSNPs can result in amino acid sequence changes, resulting in altering the function of proteins. When these nsSNPs are associated with AMR phenotype, they can act as markers for AMR (Wondji et al., 2007).
Very recently, studies have identified specific point mutations associated with phenotypic antimicrobial susceptibility of *M. bovis* in specific regions of three strains of *M. bovis* through Ilumina sequencing (Ledger et al., 2020) and on a large set of isolates for macrolides (Kinnear et al., 2020). In Kinnear et al. (2020) 100% concordance was observed between genotype (mutations in both domain II and V of the 23S rRNA) and phenotype (macrolide resistant isolates based on CLSI clinical breakpoints), showing high potential of this method to predict macrolide resistance in *M. bovis.* In human medicine, targeted sequencing has already been implemented in point-of-care settings for *M. pneumoniae* community-acquired respiratory infection outbreaks (Pereyre et al., 2016).

Limitations of present molecular genetic methods

Genetic-based antibiotic resistance determination is rapid, as in most cases it can be applied to the clinical samples, thus avoiding time losses due to isolation and identification steps. However, up until now molecular testing cannot replace phenotypic AST. An important limitation of these specific methods is the focus on known mutations in a small homogenous population. For example, the qPCR method developed by Sulyok et al. (2018) for macrolides was based on mutations at position 2059 in the 23S rRNA gene, which indeed is involved in macrolide resistance in Hungary, as well as in Japan and Canada (Sulyok et al., 2017; Hata et al., 2019; Kinnear et al., 2020 – Table 7). However, in several other countries (*e.g.* China, France, and Spain), a mutation at position 2058 is involved in macrolide resistance (Kong et al., 2016; Khalil et al., 2017; García-Galán et al., 2020), and is therefore missed by this real-time PCR method. In addition, when unknown or new resistance mechanisms, such as nsSNPs, efflux mechanisms, target-site modification by methylation, and plasmids are present, these will be overlooked (Jaillard et al., 2018). It is also not always clear whether multiple mutations or mechanisms collaborate together to obtain resistance, and it can be difficult to detect those simultaneously when only one of them is targeted by the used molecular method (Maurin, 2012). To circumvent these shortcomings, whole genome sequencing and genome-wide association studies can help to reveal both well-known and novel associations between genotype and phenotype. Even when the association between

whole genome results and phenotype remains inconclusive, it can point into the direction to investigate other resistance mechanisms (*e.g.* methylation) as well (Coll et al., 2018). Finally, in order to have clinically relevant molecular genetic assays, a causal link between genotype and phenotype should be present. Therefore clinical breakpoints should be obtained for *M. bovis* or randomized clinical trials with *M. bovis* strains of which the full genome is available.

Antimicrobial resistance mechanisms of *M. bovis*

Several AMR mechanisms have been described for different pathogens coming down to (1) preventing the antimicrobial from entering the cell (*e.g.* decreased porin formation in *Klebsiella* species), (2) efflux pumps which drift the antimicrobial out of the cell (*e.g.* efflux of tetracycline in *E. coli*), (3) inactivation by modification or degradation of the antimicrobial agent (*e.g.* beta-lactamase producing organisms), and (4) modification or blockage of the antimicrobial target (*e.g.* mutations in quinolone resistance-determining regions) (Boerlin and White, 2013). The meaning of the first three mechanisms are unclear for *M. bovis*. However, the most frequently described resistance mechanism in *M. bovis* so far, is the modification of the antimicrobial target as a result of point mutations. These mutations are able to change the protein sequences (nonsynonymous mutations) in such a way that antimicrobials cannot act on the *M. bovis* target molecule anymore.

Acquiring antimicrobial resistance

Mutations occur continuously in bacteria, and are often repaired by DNA repair mechanisms. However, due to its small genome, *M. bovis* has only few DNA repair mechanisms, resulting in higher numbers of fixed mutations (Rocha et al., 2005). The mutation frequency can increase in organisms under stress, such as chemical exposure, ultra violet light, and during antimicrobial treatment.

Other ways of acquiring AMR is through receiving DNA from other organisms (horizontal gene transfer) by (1) transformation: the uptake of DNA present in the environment, (2) transduction: injection of viral DNA by bacteriophages, and (3) conjugation: transfer of mobile genetic elements (Boerlin and White, 2013; Dordet-Frisoni et al., 2019). Well-known mobile genetic elements carrying antimicrobial resistance are plasmids, but also integrative conjugative elements (ICEs) or chromosomal conjugative transfer can spread antimicrobial resistance between bacteria. Plasmids have not yet been described in *M. bovis,* but they were abundantly found in the *Mycoplasma mycoides* cluster (Breton et al., 2012). Also the presence of a prophage, which is a bacteriophage genome integrated in the bacterial DNA, has not (yet)

been observed in *M. bovis,* but was identified in *M. agalactiae* and *M. bovigenitalium*, both part of the *hominis* cluster (Tardy et al., 2012). *Mycoplasma* integrative conjugative elements (MICEs; also conjugative transposons) are present in *M. bovis* and *M. agalaciae* (Dordet-Frisoni et al., 2014; Tardy et al., 2015), and transfer of MICE containing the *parC* and *parE* genes, which can be associated with fluoroquinolone resistance, has been observed (Dordet-Frison et al., 2014). In addition, under *in vitro* antibiotic pressure, chromosomal transfer showed to be able to transfer parts of the chromosome containing genes with specific mutations associated with enrofloxacin resistance between *M. agalactiae* isolates (Faucher et al., 2019).

In the next subchapters, the most frequently used antimicrobials for *M. bovis* infections and the associated mechanisms for acquired resistance in *M. bovis* are described in more detail.

Florfenicol

Florfenicol binds irreversibly to the 50S ribosomal subunit and inhibits protein synthesis by disturbing transpeptidation and translation of the bacterial mRNA (Dowling, 2013a; Lysnyansky and Ayling, 2016). No mutation associated with florfenicol resistance has been identified in *M. bovis* field isolates yet, but *in vitro* generated resistant mutants showed mutations in G2062T and A2063T of the 23S rRNA genes (Sulyok et al., 2017) (Table 7). However, also in field *M. ovipneumoniae* strains with increased MIC values for florfenicol, no mutations in the 23S rRNA were shown, therefore suggesting the presence of other mechanisms, such as efflux (Jaÿ et al., 2020). The *floR* gene, located on various plasmids and associated with florfenicol resistance by efflux, has been detected in *E. coli* and *Pasteurellaceae* isolated from calves (White et al., 2000; Kehrenberg and Schwarz, 2005; Katsuda et al., 2012). However, until now, no mechanism increasing the florfenicol efflux in *Mycoplasma* species has been described.

Tetracyclines

Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit and blocking tRNA to their docking site (Brodersen et al., 2000; del Castillo, 2013). Usually tetracyclines are bacteriostatic for susceptible bacteria, but for doxycycline also a bactericidal time-dependent effect has been shown (del Castillo, 2013). Doxycycline also seems to be the most effective tetracycline (lowest MIC values and non-wild type population) in the recent years (Figure 10). Probably due to a small non-wild type population, not much is known about the

resistance mechanisms (Gerchman et al., 2009; Barberio et al., 2016; Kong et al., 2016). Point mutations in the 16S rRNA-encoding genes (*rrs*1, *rrs*2) of the Tet-1 site of the 30S ribosomal subunit have been linked with tetracycline resistance in *M. bovis* (Table 7). Additionally, it seems that the more such mutations are present, the more the MIC value of these strains increases (Amram et al., 2015; Khalil et al., 2017; Sulyok et al., 2017; Hata et al., 2019).

Macrolides

Macrolides reversibly bind to the 23S rRNA of the 50S ribosomal subunit and inhibit protein synthesis by disturbing transpeptidation and translocation of bacterial mRNA (Giguère, 2013a; Lysnyansky and Ayling, 2016). They are mostly bacteriostatic, but at very high concentrations may be bactericidal (Giguère, 2013a). Resistance is caused by either target site modification through methylation or mutation, efflux pumps or enzymatic inactivation (Leclercq, 2002; Giguère, 2013a). Mutations in the 23S rRNA and L4/L22 proteins associated with macrolide resistance in *M. bovis* are shown in Table 7. The presence of methylated bases and methyltransferases have been described in several *Mycoplasma* species (Razin and Razin, 1980; Lluch-Senar et al., 2013; Wojciechowski et al., 2013), but were never associated with macrolide resistance. However, it has been shown that efflux pumps (possibly ABC-type) are involved in resistance to macrolides in *M. pneumoniae* (Li et al., 2017), but so far such efflux pumps are not identified in *M. bovis*.

Although high MIC values are reported in several studies, this does not necessarily result in therapy failure due to several features of the antimicrobials belonging to the macrolide class. Macrolides concentrate in the lung, for example tulathromycin results within 12 hours after SC administration of 2.5 mg/kg in approximately 3000 ng/g or higher for 7 days, which is much higher than in plasma (Nowakowski et al., 2004; Godinho et al., 2005a). The lung concentration was even higher for SC administration of 6 mg/kg gamithromycin, where a concentration of 18,500 ng/g was obtained after 24 hours administration, and high lung to plasma ratios (265-410) persisted over 15 days (Huang et al., 2010). This does however not explain why tulathromycin is still effective on an *M. bovis* with a MIC value of 64 µg/mL. It has also been shown that macrolides can accumulate intracellularly in phagocytic cells (Villarino et al., 2014; Lysnyansky and Ayling, 2016), just like *M. bovis* (Van Der Merwe et al., 2010; Bürki et al., 2015), and that macrolides have profitable immunomodulatory effects in humans (Zimmermann et al., 2018).

Table 7. Overview of mutations associated with antimicrobial resistance identified in *M. bovis* and for which a minimum level of evidence is available, such as presence in multiple independent isolates with increased MIC values for a relevant antimicrobial agent or a statistically significant association with phenotypic resistance. Table shows mutations ordered by gene, whenever mutations in specific genes correspond to resistance against multiple antimicrobials, this agent is pointed out by its letter between brackets (*e.g.* macrolides: (M)).

Study	Country	Method R/S [#]	*16S rRNA (rrs1 and rrs2)*	*23S rRNA (domain II/V)*	*L4*	*L22*	*gyrA*	*parC*
			Tetracyclines (T), Spectinomycine (S)	Macrolides (M), florfenicol (F), lincosamides (L)			Fluoroquinolones	
A	ISR	CLSI		G748A[b] (M)			Ser83Phe	Asp84Asn
B/C	JPN	ECOFF		G748A[b] (M)			Ser83Leu	Ser80Ile
							Ser83Phe	Ser81Pro
D	ISR, GBR, DEU	MIC		G748A[c] (M) C752T[c] (M) A2058G[c] (M) A2059G/C[c] (M)	T186P[d] (M)			
E	ISR, GBR, DEU, HUN, ESP, AUS, LIT, CUB	ECOFF	A965T[b] + A967T/C[b] (+ G1058A/C[b]) (T)					
F	CHN	CLSI		A2058G[c] (M)				
G	HUN	ECOFF	A965T[b] + A967T/C[b] (T) C1192A[a] (S)	G2062T[c] (F)* G2063T[c] (F)* G748A[b] (M) A2059G[c] (M) A2059G[c] (L)			Ser83Phe	Ser80Ile Asp84Asn
H	FRA	CLSI	A965T[c] (T) A967T[b] (T)	G748A[b] (M) A2058G[c] (M)		Q93K/H (M)		
I	JPN	MIC	A965T[c] (T) + A967T[b] (T) (+ G1058A/C[c]) (T) C1192A[c] (S)	G748A[c] (M) A2059G[c] (M) A2059G[c] (L)			Ser83Phe	Ser80
J	ESP	*PolC* type		G458A[b] (M) A2058G[c] (L)			Ser83Phe	Ser80Ile Asp84Asn
K	CAN	CLSI		G748A[c] (M) G748A[c] +A2059G[c] (M) G748A[c] +A2060G[c] (M)				

A = Lysnyansky et al., 2009; B = Sato et al., 2013; C = Sato et al., 2017; D = Lerner et al., 2014; E = Amram et al., 2015; F = Kong et al., 2016; G = Sulyok et al., 2017; H = Khalil et al., 2017; I = Hata et al., 2019; J = García-Galán et al., 2020; K = Kinnear et al., 2020; country of origin is abbreviated following ISO 3166-1; Alpha-3 code; # Method R/S: method used for categorizing isolates into resistant/susceptible (CLSI), wild type/non-wild type (ECOFF), strain type (*PolC*) or high/low MIC (MIC); CLSI = bovine respiratory pathogens or other *Mycoplasma* species, ECOFF = epidemiological cut off based on visual estimation, MIC = minimum inhibitory concentration; [a] in one alle, [b]both alleles, [c]in one or both alleles, [d]no clear association with AMR alone, * *in vitro* mutants

Lincosamides

The binding site on the 23S rRNA of the 50S subunit of lincosamides overlaps with the binding site of macrolides (Giguère, 2013b). Resistance can occur to lincosamides alone, but cross-resistance with macrolides occurs more frequently (Giguère, 2013a). Mutations in the 23S rRNA associated with lincosamide resistance are located at position 2058 and 2059 (Table 7). As far as the author is aware, no additional lincosamide resistance mechanisms have so far been described in *Mycoplasma* species.

Aminoglycosides

Aminoglycosides bind with the 30S ribosomal subunit to disturb protein synthesis (Dowling, 2013b). Whereas streptomycin only acts at one target site, the other aminoglycosides (*e.g.* neomycin, gentamicin) interfere with more sites (Dowling et al., 2013b). Plasmid-mediated enzymes are mostly responsible for resistance against aminoglycosides in many bacterial species, but such enzymes are not observed in *M. bovis* (Dowling et al., 2013b; Lysnyanski and Ayling, 2016). Mutations associated with resistance against spectinomycin are problaby the results of a single mutation in the *rrs*1 gene of the 16S rRNA at position 1192 (Sulyok et al., 2017; Hata et al., 2019) (Table 7).

Fluoroquinolones

All fluoroquinolones are antimicrobials of which the mode of operation depends on their structure, resulting in a slightly different binding affinity for their targets. These target enzymes are DNA-gyrase and topoisomerase IV (Giguère and Dowling, 2013; Redgrave et al., 2014), and resistance of *M. bovis* against fluoroquinolones is caused by alternations in the QRDRs of DNA gyrase (*gyrA* and *gyrB* genes) and/or topoisomerase IV subunits (*parC* and *parE*) (Gautier-Bouchardon, 2018). Mutations associated with increased MIC values are shown in Table 7. The accumulation of different mutations appears to result in step-wise resistance (Lysnyansky et al., 2009, Sato et al., 2013; Hata et al., 2019). Efflux pumps have been identified in *M. hominis* by Raherison et al. (2002), and were also suggested to be present in *M. mycoides subsp. capri* (Antunes et al., 2015), but this has not been investigated for *M. bovis*.

Table 7 shows an overview of mutations identified in *M. bovis* which were either found in multiple isolates with increased MIC values or were associated with AMR supported by statistical analysis. Besides those mutations, many more mutations were identified in DNA

regions associated with AMR, but not (yet) evidently associated with AMR in *M. bovis*, and are therefore not included in Table 5.

Antimicrobial susceptibility of *M. bovis*

An overall decrease in the antimicrobial susceptibility of *M. bovis* to various antimicrobial classes targeting protein synthesis (*e.g.* phenicols, tetracyclines, lincosamides and macrolides) and DNA synthesis (*e.g.* fluoroquinolones) has been reported in many countries (Gautier-Bouchardon et al., 2014; Cai et al., 2019; Klein et al., 2019). For example, susceptibility in France decreased between 1978-1979 and 2010-2012 for eight antimicrobial agents within the classes of the tetracyclines, fluoroquinolones, aminoglycosides and macrolides (Gautier-Bouchardon et al., 2014). In Canada, an increased MIC_{50} was seen for tetracyclines, and tylosin over two decades, and remained high in the third decade (Cai et al., 2019). Klein et al. (2019) demonstrated a slight increase in MIC_{50} (at most one doubling dilution) between isolates obtained in Europe between 2014-2017 and 2010-2012, for almost all antimicrobials. Only for oxytetracycline a small reduction was observed (Klein et al., 2019). Especially high MIC_{50} and MIC_{90} values against tetracyclines and macrolides have been reported, as shown in Figure 12. In most countries the major part of *M. bovis* isolates are still susceptible to fluoroquinolones (Cai et al., 2019; Becker et al., 2020; Liu et al., 2020), although high MIC_{50-90} have been reported for Italian and Spanish strains (Klein et al., 2019; Garciá-Galan et al., 2020). Nevertheless, fluoroquinolones are critically important antibiotics for human medicine, and therefore recommended to be only used in food-producing animals, when susceptibility testing shows resistance against all other antimicrobial classes (WHO, 2017). More recently, macrolides were also added to this list, leaving the use of florfenicol and tetracyclines as good first choice treatment options in some countries, but problematic for countries with high percentages of resistant *M. bovis* against these drugs, such as France (Becker et al., 2015).

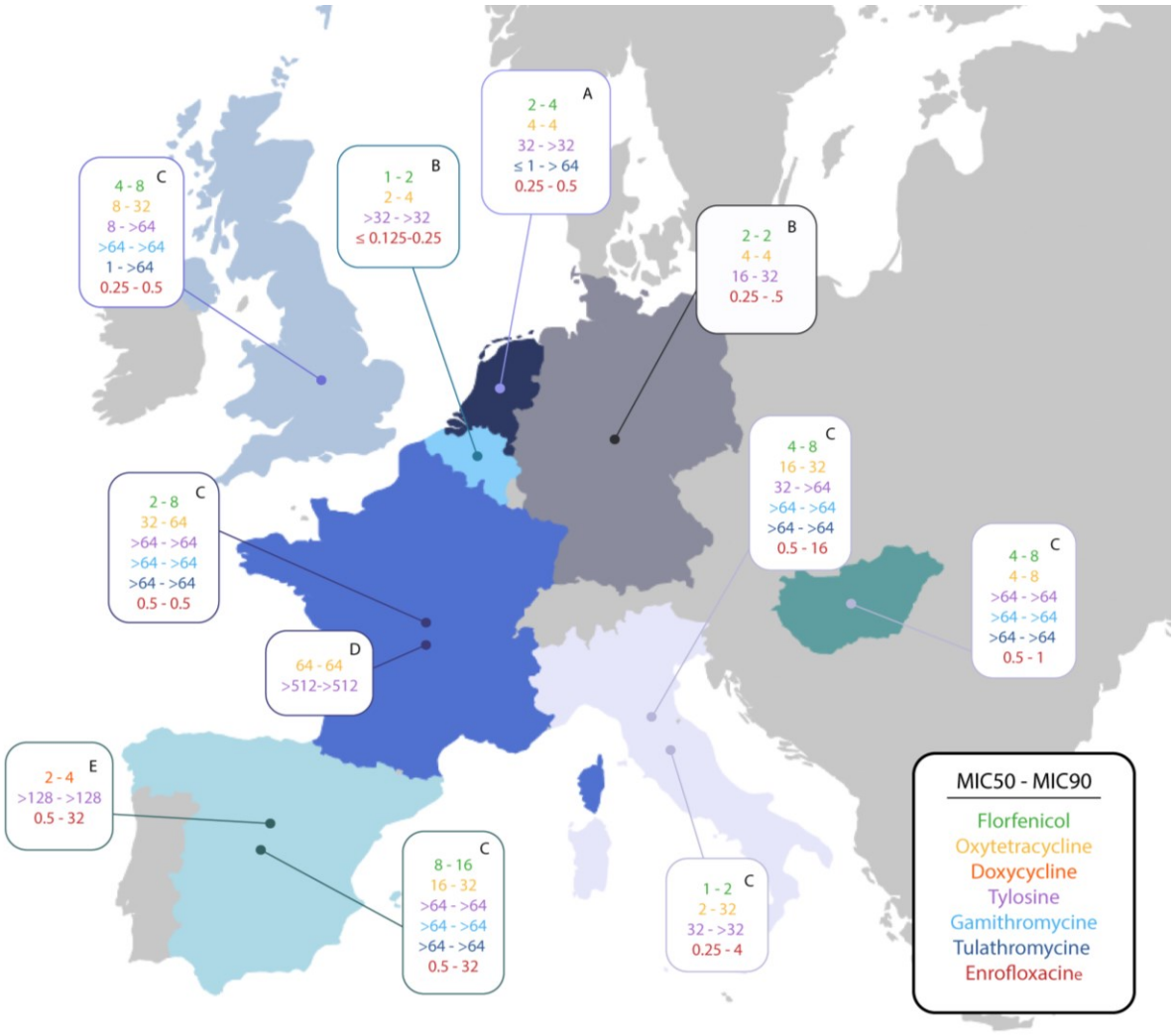
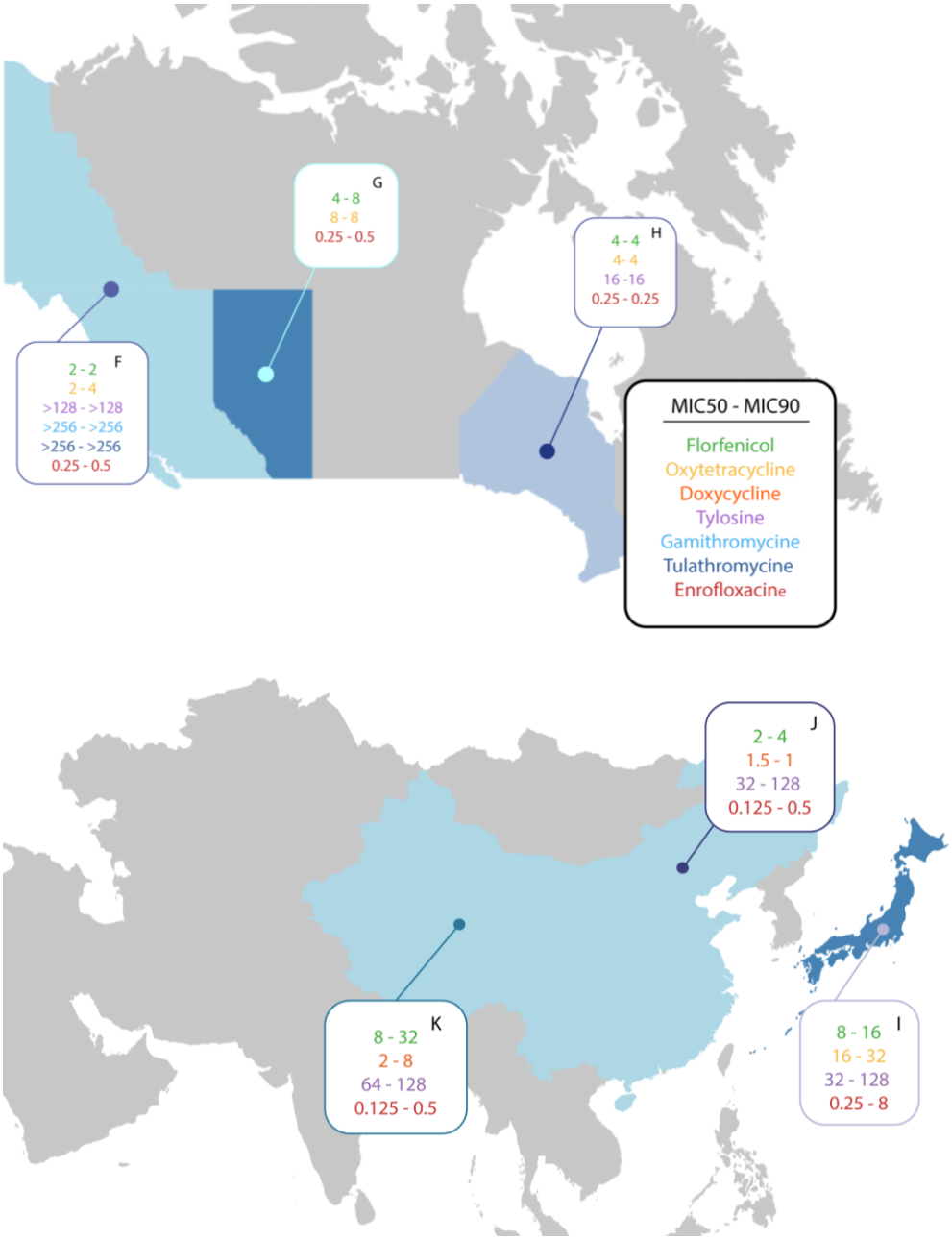


Figure 12. Maps of Europe (a), Canada (b), China and Japan (c), showing reported MIC_{50} and MIC_{90} (µg/mL) values for different antimicrobials (corresponding to specific legend color) since 2015. MIC_{50} and MIC_{90} for tilmicosin was excluded from this figure, as both $MIC_{50\text{-}90}$ were generally high in all studies (>32 µg/mL). Letters are corresponding to the different studies: A: Heuvelink et al., 2016; B: Barberio et al., 2016; C: Klein et al., 2019; D: Khalil et al., 2017; E: García-Galán et al., 2020; F: Jelinski et al., 2020; G: Anholt et al., 2017; H: Cai et al., 2019; I: Hata et al., 2019, J: Kong et al., 2016; K: Liu et al., 2020. Maps were created using Piktochart (2020, www.piktochart.com).



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Differences in MIC values for *M. bovis* can be observed due to year or site of isolation, type of livestock production systems, age or health status of livestock, geographical origin, and antimicrobial treatment history (Gerchman et al., 2009; Siugzdaite et al., 2012; Heuvelink et al., 2016; Lysnyansky and Ayling, 2016; Gautier-Bouchardon et al., 2018; Cai et al., 2019; Jelinski et al., 2020). There are also studies where no significant association between site of isolation, geographic location, breed of cattle, antimicrobial use or strain type were evidenced (Francoz et al., 2005; Barberio et al., 2016; Khalil et al., 2017; Cai et al., 2019; Becker et al., 2020).

M. bovis isolates with acquired resistance against certain antimicrobials (*e.g.* tetracycline, valnemulin) have been described to regain susceptibility after a short time in the absence of the antimicrobial *in vitro* (Sulyok et al., 2017). This is in contrast to fluoroquinolones, as Sulyok et al. (2017) tested three *M. bovis* isolates *in vitro* which remained resistant for several passages without fluoroquinolone presence. Also the frequency in which resistance of *M. bovis* against antimicrobials develops seem to differ between antimicrobial agents. For example resistance against florfenicol and tiamulin developed more rapid *in vitro* in three *M. bovis* isolates than resistance against tetracyclines (Sulyok et al., 2017). Cross-resistance may also occur between tetracyclines, macrolides (tylosin and tilmicosin), tiamulin and florfenicol, among fluoroquinolones (enrofloxacin, danofloxacin, marbofloxacin), and also between macrolides, pleuromutilins, and lincosamides (Sulyok et al., 2017; Cai et al., 2019). However, again, we should be cautious translating these results to clinical relevance. Most of the AMR in these studies developed *in vitro*, and as AMR mechanisms by mutation can interfere with bacterial virulence and fitness (Beceiro et al., 2013), it is not clear whether the observations described above are relevant *in vivo.*

Important drivers associated with antimicrobial resistance

It is important to understand the drivers behind AMR of *M. bovis* in order to prevent further AMR development, and ideally to reduce AMR of *M. bovis* and other pathogens. The intensive use of antimicrobials is most likely the largest driver for the development of AMR (Barbosa and Levy, 2000; Holmes et al., 2016). Administration of antimicrobials can result in inhibiting the growth or killing the susceptible population of both the targeted pathogen and the commensal microbiota. Subsequently, the resistant population that survives, can multiply in the host or can be transmitted to the environment (Barbosa and Levy, 2000).

M. bovis is inherently resistant against many agents used as first choice antimicrobials for treating BRD, such as penicillin and potentiated sulphonamides. The use of these antimicrobials will therefore mainly affect the commensal microbiota or potentially secondary pathogens, while leaving *M. bovis* unharmed. When treated with antimicrobials effective against *M. bovis* (*e.g.* tetracyclines, macrolides, ..), both *M. bovis* and the commensal microbiota are exposed to an antimicrobial selective pressure.

Next to the choice of antimicrobial drug, the administered dose, treatment duration and treatment interval are very important in the development of AMR as these influence the course of serum or tissue drug concentration over time (Catry et al., 2003). It has been hypothesized, "that for each antimicrobial-pathogen combination and antimicrobial concentration a range exists in which selective amplification of single-step, drug-resistant mutants occur". This range is called the 'mutant selection window' (MSW) (Drlica and Zhao, 2007). Every time a drug is administered, this MSW will be passed at least twice, resulting in selection pressure on the population. Starting with high concentrations, and repeating the treatment before the MSW is reached, will prevent extensive AMR selection (Drlica, 2003). This MSW is believed to be located between the MIC and 'mutant prevention concentration' (MPC) ('the minimum concentration that inhibits growth of the least-susceptible single-step mutant subpopulation', Huang et al., 2020). The duration of the serum or tissue drug concentrations being in the MSW, is for example depending on maximum plasma concentrations, time of peak concentration, distribution volume, and half-life of the antimicrobial (Catry et al., 2003). For *M. hyopneumoniae* and *M. gallisepticum,* the existence of a MSW for tetracyclines, macrolides, pleuromutilins and fluoroquinolones was explored (Zhang et al., 2017; Huang et al., 2020). The analyses showed that danofloxacin, macrolides (tylosin, tilmicosin), and doxycycline are more likely to select resistant mutants than pleuromutilins (tiamulin, valnemulin) in both *Mycoplasma* species. Unfortunately, no such studies have been performed for *M. bovis* so far.

In outbreaks of *M. bovis*, metaphylactic treatment is often used (as described above). Group treatment compared to individual treatment, increases the selection pressure, and is most likely associated with increased AMR (Dunlop et al., 1998; Varga et al., 2009; Graveland et al., 2010). However, these studies did not take administration route into account, which can be a confounder as different routes of administration may lead to different course of serum and tissue concentrations, resulting in a variety of duration in the MSW (Wiuff et al., 2002;

Catry et al., 2003). However, oral group treatment in veal calves was associated with increased AMR in BRD pathogens (*M. haemolytica*, *P. multocida*) and *E. coli* compared to individual parenteral treatment (Schönecker et al., 2019). Although antimicrobials in feed as growth promoter are prohibited in Europe, and not commonly used for metaphylactic treatment in dairy and beef herds, the use of oral group treatment is still widely used in the veal calf sector in for example France, Belgium and The Netherlands, when facing *M. bovis* outbreaks (Jarrige et al., 2017; Bokma et al., 2019a; SDa, 2020). This is in contrast to other countries, such as Denmark and Switzerland, which already made a switch towards more individual treatment (Fertner et al., 2016; Lava et al., 2016). Oral group treatment is a risk for AMR in both pathogens as the commensal microbiota, and appropriate individual treatment of calves is necessary to reduce AMR (Schönecker et al., 2019).

To be able to start appropriate individual antimicrobial treatment in calves facing *M. bovis*, rapid identification of this pathogen is necessary to prevent the use of ineffective antimicrobials (*e.g.* penicillin, (potentiated) sulphonamides), and to reduce unnecessary antimicrobial pressure on commensal flora. Subsequently, antimicrobial susceptibility testing could be an important tool to prevent further stimulation of acquired antimicrobial resistance in *M. bovis* and therapy failure. In addition, strain typing of the isolate can support in control and prevention measurements by showing insights in transmission and virulence of the circulating *M. bovis* strain(s).

REFERENCES

Adegboye, D.S., Halbur, P.G., Cavanaugh, D.L., Werdin, R.E., Chase, C.C.L., Miskimins, D.W., Rosenbusch, R.F. 1995. Immunohistochemical and pathological study of *Mycoplasma bovis*-associated lung abscesses in calves. *J Vet Diagnostic Investig* 7, 333-7.

Aebi, M., Bodmer, M., Frey, J., Pilo, P. 2012. Herd-specific strains of *Mycoplasma bovis* in outbreaks of mycoplasmal mastitis and pneumonia. *Vet Microbiol 157*, 363-368.

Aebi, M., van den Borne, B.H.P., Raemy, A., Steiner, A., Pilo, P., Bodmer, M. 2015. *Mycoplasma bovis* infections in Swiss dairy cattle: A clinical investigation. *Acta Vet Scand 57*, 10.

Ahani Azari, A., Amanollahi, R., Jafari Jozani, R., Trott, D.J., Hemmatzadeh, F. 2020. High-resolution melting curve analysis: a novel method for identification of *Mycoplasma* species isolated from clinical cases of bovine and porcine respiratory disease. *Trop Anim Health Prod 52*, 1043-1047.

Aidara-Kane, A., Angulo, F.J., Conly, J., Minato, Y., Silbergeld, E.K., McEwen, S.A., Collignon, P.J., Balkhy, H., Collignon, P., Friedman, C., Hollis, A., Kariuki, S., Kwak, H.S., McEwen, S., Moulin, G., Ngandjio, A., Rollin, B., Rossi, F., Wallinga, D. 2018. World Health Organization (WHO) guidelines on use of medically important antimicrobials in food-producing animals. *Antimicrob Resist Infect Control 7*, 7.

Al-Farha, A.A.B., Hemmatzadeh, F., Khazandi, M., Hoare, A., Petrovski, K. 2017. Evaluation of effects of *Mycoplasma* mastitis on milk composition in dairy cattle from South Australia. *BMC Vet Res 13*, 351.

Alabdullah, A. 2017. *Mycoplasma bovis* associated diseases: modeling stress and its effect on bovine immune function. Washington State University. College of Veterinary Medicine, Washington, United States of America.

Alabdullah, H.A., Fox, L.K., Gay, J.M., Barrington, G.M. 2018. Interactive effects of dexamethasone and opsonized *Mycoplasma bovis* on bovine neutrophil function in vitro. *Vet Immunol Immunopathol 196*, 18-21.

Alabdullah, H.A., Fox, L.K., Gay, J.M., Barrington, G.M., Mealey, R.H. 2015. Effects of dexamethasone and *Mycoplasma bovis* on bovine neutrophil function in vitro. *Vet Immunol Immunopathol 164*, 67-73.

Alberti, A., Addis, M.F., Chessa, B., Cubeddu, T., Profiti, M., Rosati, S., Ruiu, A., Pittau, M. 2006. Molecular and antigenic characterization of a *Mycoplasma bovis* strain causing an outbreak of infectious keratoconjunctivitis. *J Vet Diagn Invest 18*, 41-51.

Amram, E., Freed, M., Khateb, N., Mikula, I., Blum, S., Spergser, J., Sharir, B., Ozeri, R., Harrus, S., Lysnyansky, I. 2013. Multiple locus variable number tandem repeat analysis of *Mycoplasma bovis* isolated from local and imported cattle. *Vet J 197,* 286-290.

Amram, E., Mikula, I., Schnee, C., Ayling, R.D., Nicholas, R.A.J., Rosales, R.S., Harrus, S.,

Lysnyanskya, I. 2015. 16S rRNA gene mutations associated with decreased susceptibility to tetracycline in *Mycoplasma bovis*. *Antimicrob Agents Chemother 59,* 796-802.

Anderson, N.W., Buchan, B.W., Riebe, K.M., Parsons, L.N., Gnacinski, S., Ledeboer, N.A. 2012. Effects of solid-medium type on routine identification of bacterial isolates by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol 50*, 1008-1013.

Andersson, A.M., Aspán, A., Wisselink, H.J., Smid, B., Ridley, A., Pelkonen, S., Autio, T., Lauritsen, K.T., Kensø, J., Gaurivaud, P., Tardy, F. 2019. A European inter-laboratory trial to evaluate the performance of three serological methods for diagnosis of *Mycoplasma bovis* infection in cattle using latent class analysis. *BMC Vet Res 15*, 369.

Anholt, R.M., Klima, C., Allan, N., Matheson-Bird, H., Schatz, C., Ajitkumar, P., Otto, S.J.G., Peters, D., Schmid, K., Olson, M., McAllister, T., Ralston, B. 2017. Antimicrobial susceptibility of bacteria that cause bovine respiratory disease complex in Alberta, Canada. *Front Vet Sci 4*, 207.

Animal and Plant Health Agency. 2016. Provision of requested information, reference ATIC0815. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/508811/ATIC0815.pdf (last accessed on March 10th 2021).

Antunes, N.T., Assunção, P., Poveda, J.B., Tavío, M.M. 2015. Mechanisms involved in quinolone resistance in *Mycoplasma mycoides* subsp. *capri*. *Vet J 204*, 327-332.

Arcangioli, M.A., Aslan, H., Tardy, F., Poumarat, F., Le Grand, D. 2012. The use of pulsed-field gel electrophoresis to investigate the epidemiology of *Mycoplasma bovis* in French calf feedlots. *Vet J 192*, 96-100.

Arcangioli, M.A., Duet, A., Meyer, G., Dernburg, A., Bézille, P., Poumarat, F., Le Grand, D. 2008. The role of *Mycoplasma bovis* in bovine respiratory disease outbreaks in veal calf feedlots. *Vet J 177,* 89-93.

Askaa, G., Erno, H. 1976. Elevation of *Mycoplasma agalactiae* subsp. *bovis* to species rank: *Mycoplasma bovis* (Hale et al). comb. nov. *Int J Syst Bacteriol 26,* 323-325.

Azarian, T., Cook, R.L., Johnson, J.A., Guzman, N., McCarter, Y.S., Gomez, N., Rathore, M.H., Morris, J.G., Salemi, M. 2015. Whole-Genome Sequencing for outbreak investigations of Methicillin-Resistant *Staphylococcus aureus* in the neonatal intensive care unit: time for routine practice. *Infect Control Hosp Epidemiol 36*, 777-785.

Baggott, D., Casartelli, A., Fraisse, F., Manavella, C., Marteau, R., Rehbein, S., Wiedemann, M., Yoon, S. 2011. Demonstration of the metaphylactic use of gamithromycin against bacterial pathogens associated with bovine respiratory disease in a multicentre farm trial. *Vet Rec 168,* 241.

Baptiste, K.E., Kyvsgaard, N.C. 2017. Do antimicrobial mass medications work? A systematic review and meta-analysis of randomised clinical trials investigating antimicrobial prophylaxis or metaphylaxis against naturally occurring bovine respiratory disease. *Pathog Dis 75*, ftx083.

Barberio, A., Flaminio, B., De Vliegher, S., Supré, K., Kromker, V., Garbarino, C., Arrigoni, N., Zanardi, G., Bertocchi, L., Gobbo, F., Catania, S., Moroni, P. 2016. Short communication: In vitro antimicrobial susceptibility of *Mycoplasma bovis* isolates identified in milk from dairy cattle in Belgium, Germany, and Italy. *J Dairy Sci 99*, 6578-6584.

Barbosa, T.M., Levy, S.B. 2000. The impact of antibiotic use on resistance development and persistence. *Drug Resist. Updat 3*, 303-311.

Bartram, D.J., Moyaert, H., Vanimisetti, B.H., Ramage, C.P., Reddick, D., Stegemann, M.R. 2016. Comparative efficacy of tulathromycin and tildipirosin for the treatment of experimental *Mycoplasma bovis* infection in calves. *Vet Med Sci 2*, 170-178.

Beceiro, A., Tomás, M., Bou, G. 2013. Antimicrobial resistance and virulence: A successful or deleterious association in the bacterial world? *Clin Microbiol Rev 26,* 185-230.

Becker, C.A.M., Ambroset, C., Huleux, A., Vialatte, A., Colin, A., Tricot, A., Arcangioli, M.A., Tardy, F. 2020. Monitoring *Mycoplasma bovis* diversity and antimicrobial susceptibility in calf feedlots undergoing a respiratory disease outbreak. *Pathogens 9*, 593.

Becker, C.A.M., Thibault, F.M., Arcangioli, M.A., Tardy, F. 2015. Loss of diversity within *Mycoplasma bovis* isolates collected in France from bovines with respiratory diseases over the last 35years. *Infect Genet Evol 33*, 118-126.

Ben Shabat, M., Mikula, I., Gerchman, I., Lysnyansky, I. 2010. Development and evaluation of a novel single-nucleotide-polymorphism real-time PCR assay for rapid detection of fluoroquinolone-resistant Mycoplasma bovis. *J Clin. Microbiol 48*, 2909-2915.

Bennett, R.H., Jasper, D.E., 1980. Bovine mycoplasma mastitis from intramammary inoculation of small numbers of *Mycoplasma bovis*: local and systematic antibody response. *Am J Vet Res* 41, 889-892.

Besser, J., Carleton, H.A., Gerner-Smidt, P., Lindsey, R.L., Trees, E. 2018. Next-generation sequencing technologies and their application to the study and control of bacterial infections. *Clin Microbiol Infect* 24, 335–341.

Bicknell, S.R., Gunning, R.F., Jackson, G., Boughton, E., Wilson, C.D. 1983. Eradication of *Mycoplasma bovis* infection from a dairy herd in Great Britain. *Vet Rec 112*, 294-297.

Biddle, M.K., Fox, L.K., Evans, M.A., Gay, C.C. 2005. Pulsed-field gel electrophoresis patterns of *Mycoplasma* isolates from various body sites in dairy cattle with Mycoplasma mastitis. *J Am Vet Med Assoc 227*, 455-459.

Biddle, M.K., Fox, L.K., Hancock, D.D., Gaskins, C.T., Evans, M.A. 2004. Effects of storage time and thawing methods on the recovery of *Mycoplasma* species in milk samples from cows with intramammary infections. *J Dairy Sci 87,* 933-936.

Bizzini, A., Greub, G. 2010. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. *Clin Microbiol Infect 16*, 1614-1619.

Boerlin, P., White, D.G. 2013. Antimicrobial resistance and its epidemiology. Antimicrobial Therapy in Veterinary Medicine, Fifth Edition. Edited by Steeve Giguère, John F. Prescott and Patricia M. Dowling.

Bokma, J., Boone, R., Deprez, P., Pardon, B. 2019a. Risk factors for antimicrobial use in veal calves and the association with mortality. *J Dairy Sci 102,* 607-618.

Bokma, J., van Leenen, K., De Cremer, L., Boyen, F., Pardon, B. 2019b. *Mycoplasma bovis* peritonitis and omental bursitis after C-section in two Belgian Blue cows. In Programme: European bovine congress 2019 : your veterinary toolbox 2025. 's Hertogenbosch, The Netherlands.

Booker, C.W., Lubbers, B. V. 2020. Bovine Respiratory Disease Treatment Failure: Impact and Potential Causes. *Vet Clin North Am Food Anim Pract 36,* 487-496.

Boonyayatra, S., Fox, L.K., Besser, T.E., Sawant, A., Gay, J.M. 2010. Effects of storage methods on the recovery of *Mycoplasma* species from milk samples. *Vet Microbiol 144,* 210-213.

Boonyayatra, S., Fox, L.K., Gay, J.M., Sawant, A., Besser, T.E. 2012. Discrimination between *Mycoplasma* and *Acholeplasma* species of bovine origin using digitonin disc diffusion assay, nisin disc diffusion assay, and conventional polymerase chain reaction. *J Vet Diagn Invest 24,* 7-13.

Boothby, J.T., Jasper, D.E., Zinkl, J.G., Thomas, C.B., Dellinger, J.D. 1983. Prevalence of mycoplasmas and immune responses to *Mycoplasma bovis* in feedlot calves. *Am J Vet Res 44*, 831-838.

Boucher, S., Gracia, E., Villa, A., Fernández, A., Nouaille, L., Briffaud, M.A., Albizu, I., Baselga, R. 2001. Pathogens in the reproductive tract of farm rabbits. *Vet Rec* 149, 677-678.

Bourassa, L., Butler-Wu, S.M. 2015. MALDI-TOF mass spectrometry for microorganism identification, in: Methods in Microbiology.

Bourdon, N., Bérenger, R., Lepoultier, R., Mouet, A., Lesteven, C., Borgey, F., Fines-Guyon, M., Leclercq, R., Cattoir, V. 2010. Rapid detection of vancomycin-resistant enterococci from rectal swabs by the Cepheid Xpert vanA/vanB assay. *Diagn Microbiol Infect Dis 67,* 291-293.

Bras, A.L., Barkema, H.W., Woodbury, M., Ribble, C., Perez-Casal, J., Windeyer, M.C. 2016. Risk factors for *Mycoplasma bovis*-associated disease in farmed bison (Bison bison) herds in western Canada: A case-control study. *Prev Vet Med 129,* 67-73.

Bras, A.L., Barkema, H.W., Woodbury, M.R., Ribble, C.S., Perez-Casal, J., Windeyer, M.C. 2017. Clinical presentation, prevalence, and risk factors associated with *Mycoplasma bovis*–associated disease in farmed bison (Bison bison) herds in western Canada. *J Am Vet Med Assoc 250,* 1167-1175.

Breton, M., Tardy, F., Dordet-Frisoni, E., Sagne, E., Mick, V., Renaudin, J., Sirand-Pugnet, P., Citti, C., Blanchard, A. 2012. Distribution and diversity of mycoplasma plasmids: Lessons from cryptic genetic elements. *BMC Microbiol 12*, 257.

Brodersen, D.E., Clemons, W.M., Carter, A.P., Morgan-Warren, R.J., Wimberly, B.T., Ramakrishnan,

V. 2000. The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B, on the 30S ribosomal subunit. *Cell 103*, 1143-1154.

Bryson, D.G., Ball, H.J., Brice, N., Forster, F., Pollock, D.S. 1999. Pathology of induced *Mycoplasma bovis* calf pneumonia in experimentally vaccinated animals. In: Stipkovits, L., Rosengarten, R., Frey, J. Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics. Vol 3. Brussels, Belgium: European Commission, 14-17.

Buchenau, I., Poumarat, F., Grand, D. Le, Linkner, H., Rosengarten, R., Hewicker-Trautwein, M. 2010. Expression of *Mycoplasma bovis* variable surface membrane proteins in the respiratory tract of calves after experimental infection with a clonal variant of *Mycoplasma bovis* type strain PG45. *Res Vet Sci 89*, 223-229.

Bürki, S., Gaschen, V., Stoffel, M.H., Stojiljkovic, A., Frey, J., Kuehni-Boghenbor, K., Pilo, P. 2015. Invasion and persistence of *Mycoplasma bovis* in embryonic calf turbinate cells. *Vet Res 46*, 53.

Bürki, S., Spergser, J., Bodmer, M., Pilo, P. 2016. A dominant lineage of *Mycoplasma bovis* is associated with an increased number of severe mastitis cases in cattle. *Vet Microbiol 196*, 63-66.

Burnens, A.P., Bonnemain, P., Bruderer, U., Schalch, L., Audigé, L., Le Grand, D., Poumarat, F., Nicolet, J. 1999. Study on the seroprevalence of infections by *Mycoplasma bovis* in Swiss cattle including epidemiological analysis of risk factors in a local population (Jura region). *Schweiz Arch Tierheilkd141*, 455-460.

Butler, J.A., Pinnow, C.C., Thomson, J.U., Levisohn, S., Rosenbusch, R.F. 2001. Use of arbitrarily primed polymerase chain reaction to investigate *Mycoplasma bovis* outbreaks. *Vet Microbiol 78,* 175-181.

Butler, J.A., Sickles, S.A., Johanns, C.J., Rosenbusch, R.F. 2000. Pasteurization of discard mycoplasma mastitic milk used to feed calves: Thermal effects on various mycoplasma. *J Dairy Sci 83,* 2285-2288.

Byrne, W., Markey, B., McCormack, R., Egan, J., Ball, H., Sachse, K. 2005. Persistence of *Mycoplasma bovis* infection in the mammary glands of lactating cows inoculated experimentally. *Vet Rec 156*, 767-771.

Cai, H.Y., McDowall, R., Parker, L., Kaufman, E.I., Caswell, J.L. 2019. Changes in antimicrobial susceptibility profiles of *Mycoplasma bovis* over time. *Can J Vet Res 83*, 34-41.

Calcutt, M.J., Lysnyansky, I., Sachse, K., Fox, L.K., Nicholas, R.A.J., Ayling, R.D. 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. *Transbound Emerg Dis 65,* 91-109.

Callens, B., Dewulf, J., Kronvall, G., Catry, B., Haesebrouck, F., Boyen, F. 2016. Antimicrobial resistance surveillance in *Escherichia coli* by using normalized resistance interpretation. *Vet Microbiol 197,* 1-7.

Castillo-Alcala, F., Bateman, K.G., Cai, H.Y., Schott, C.R., Parker, L., Clark, M.E., McRaild, P., McDowall, R.M., Foster, R.A., Archambault, M., Caswell, J.L. 2012. Prevalence and genotype

of *Mycoplasma bovis* in beef cattle after arrival at a feedlot. *Am J Vet Res 73,* 1932-1943.

Caswell, J.L., Archambault, M. 2007. *Mycoplasma bovis* pneumonia in cattle. *Anim Health Res Rev 8*, 161-86.

Caswell, J.L., Bateman, K.G., Cai, H.Y., Castillo-Alcala, F. 2010. *Mycoplasma bovis* in respiratory disease of feedlot cattle. *Vet Clin North Am Food Anim Pract 26,* 365-379.

Catry, B., Duchateau, L., Van De Ven, J., Laevens, H., Opsomer, G., Haesebrouck, F., De Kruif, A. 2008. Efficacy of metaphylactic florfenicol therapy during natural outbreaks of bovine respiratory disease. *J Vet Pharmacol Ther 31,* 479-487.

Catry, B., Laevens, H., Devriese, L.A., Opsomer, G., De Kruif, A. 2003. Antimicrobial resistance in livestock. *J Vet Pharmacol Ther 26,* 81-93.

Ceyssens, P.J., Soetaert, K., Timke, M., Bossche, A. Van Den, Sparbier, K., De Cremer, K., Kostrzewa, M., Hendrickx, M., Mathys, V. 2017. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for combined species identification and drug sensitivity testing in mycobacteria. *J Clin Microbiol 55*, 624-634.

Chase, C., Kaushik, R.S. 2019. Mucosal immune system of cattle: all immune responses begin here. *Vet Clin North Am Food Anim Pract 35*, 431-451.

Chima, J.C., Wilkie, B.N., Ruhnke, H.L., Truscott, R.B., Curtis, R.A. 1980. Immunoprophylaxis of experimental *Mycoplasma bovis* arthritis in calves. Protective efficacy of live organisms and formalinized vaccines. *Vet Microbiol 5*, 113-122.

Clark, A.E., Kaleta, E.J., Arora, A., Wolk, D.M. 2013. Matrix-Assisted laser desorption ionization-time of flight mass spectrometry: A fundamental shift in the routine practice of clinical microbiology. *Clin Microbiol Rev 26,* 547-603.

Coll, F., Phelan, J., Hill-Cawthorne, G.A., Nair, M.B., Mallard, K., Ali, S., Abdallah, A.M., Alghamdi, S., Alsomali, M., Ahmed, A.O., Portelli, S., Oppong, Y., Alves, A., *et al*. 2018. Genome-wide analysis of multi- and extensively drug-resistant *Mycobacterium tuberculosis*. *Nat Genet 50*, 307-316.

Cornelissen, J.B.W.J., de Bree, F.M., van der Wal, F.J., Kooi, E.A., Koene, M.G.J., Bossers, A., Smid, B., Antonis, A.F., Wisselink, H.J. 2017. *Mycoplasma* detection by triplex real-time PCR in bronchoalveolar lavage fluid from bovine respiratory disease complex cases. *BMC Vet Res 13,* 97.

Costard, S., Wieland, B., De Glanville, W., Jori, F., Rowlands, R., Vosloo, W., Roger, F., Pfeiffer, D.U., Dixon, L.K. 2009. African swine fever: How can global spread be prevented? *Philos Trans R Soc B Biol Sci 364,* 2683-2696.

Currin, J.F. 2009. Mycoplasma in beef cattle. Virginia Co-operative Extension. Communications and Marketing, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University. https://www.pubs.ext.vt.edu/content/dam/pubs_ext_vt_edu/400/400-304/400-304_pdf.pdf

Davies, N.G., Abbott, S., Barnard, R.C., Jarvis, C.i., Kucharski, A.J., Munday, J., Perason, C.A.B., Russel, T.W., Tully, D.C., Washburne, A.D., Wenseleers, T., Gimma, A., Waites, W., Wong, K.L.M., van Zandvoort, K., Silverman, J.D., CMMID Covid-19 Working group, Diaz-Ordaz, K., Keogh, R., Eggo, R.M., Funk, S., Jit, M. Atkins, K.E., Edumunds, W.J. 2020. Estimated transmissibility and severity of novel SARS-CoV-2 Variant of Concern 202012/01 in England. *medRXiv*, preprint.

De Cremer, L., van Leenen, K., Van Driessche, L., Bokma, J., Deprez, P., Pardon, B. 2019. Lung ultrasonography to reduce antimicrobial therapy length in an outbreak of *Mycoplasma bovis*. European Bovine Congress, 11-13 September, 's-Hertogenbosch, the Netherlands.

Dedonder, K.D., Apley, M.D. 2015. A literature review of antimicrobial resistance in Pathogens associated with bovine respiratory disease. *Anim Heal Res Rev 16,* 125-134.

Del Castillo, J.R.E. 2013. Chapter 15. Tetracyclines. Antimicrobial Therapy in Veterinary Medicine, Fifth Edition. Edited by Steeve Giguère, John F. Prescott and Patricia M. Dowling.

Deng, X., den Bakker, H.C., Hendriksen, R.S. 2016. Genomic epidemiology: Whole-Genome-Sequencing–powered surveillance and outbreak investigation of foodborne bacterial pathogens. *Annu Rev Food Sci Technol 7,* 353-374.

Devriese, L.A., Haesebrouck, F. 1991. Antibiotic susceptibility testing of *Mycoplasma bovis* using Tween 80 hydrolysis as an indicator of growth. *Zentralbl Veterinarmed B 38*, 781-783.

Dingle, T.C., Butler-Wu, S.M. 2013. MALDI-TOF mass spectrometry for microorganism identification. *Clin Lab Med 33*, 589-609.

Dordet-Frisoni, E., Faucher, M., Sagné, E., Baranowski, E., Tardy, F., Nouvel, L.X., Citti, C. 2019. Mycoplasma chromosomal transfer: A distributive, conjugative process creating an infinite variety of mosaic genomes. *Front Microbiol 10*, 2441.

Dordet-Frisoni, E., Sagné, E., Baranowski, E., Breton, M., Nouvel, L.X., Blanchard, A., Marenda, M.S., Tardy, F., Sirand-Pugnet, P., Citti, C. 2014. Chromosomal transfers in mycoplasmas: When minimal genomes go mobile. *MBio 5*, e01958.

Dowling, P.M. 2013a. Chloramphenicol, Thiamphenicol, and Florfenicol, in: Antimicrobial Therapy in Veterinary Medicine.

Dowling, P.M. 2013b. Aminoglycosides and Aminocyclitols, in: Antimicrobial Therapy in Veterinary Medicine.

Doyle, D., Credille, B., Lehenbauer, T.W., Berghaus, R., Aly, S.S., Champagne, J., Blanchard, P., Crossley, B., Berghaus, L., Cochran, S., Woolums, A. 2017. Agreement among 4 sampling methods to identify respiratory disease. *J Vet Intern Med 31*, 954-959.

Drlica, K. 2003. The mutant selection window and antimicrobial resistance. *J Antimicrob Chemother 52*, 11-7.

Drlica, K., Zhao, X. 2007. Mutant selection window hypothesis updated. *Clin Infect Dis 44*, 681-688.

Dudek, K., Bednarek, D., Ayling, R.D., Kycko, A., Reichert, M. 2019. Preliminary study on the

effects of enrofloxacin, flunixin meglumine and pegbovigrastim on *Mycoplasma bovis* pneumonia. *BMC Vet Res 15*, 371.

Dudek, K., Bednarek, D., Ayling, R.D., Kycko, A., Szacawa, E., Karpińska, T.A. 2016. An experimental vaccine composed of two adjuvants gives protection against *Mycoplasma bovis* in calves. *Vaccine 34,* 3051-3058.

Dudek, K., Bednarek, D., Ayling, R.D., Szczotka, M., Iwan, E., Kocki, J. 2018. Analysis of the immune response of calves to various saponin-based adjuvants for an experimental *Mycoplasma bovis* vaccine. *Acta Vet Hung 66,* 226-240.

Dudek, K., Nicholas, R.A.J., Szacawa, E., Bednarek, D. 2020. *Mycoplasma bovis* infections—Occurrence, diagnosis and control. *Pathogens 9*, 640.

Dudek, K., Szacawa, E., Nicholas, R.A.J. 2021. Recent developments in vaccines for bovine Mycoplasmoses caused by *Mycoplasma bovis* and *Mycoplasma mycoides* subsp. *mycoides*. *Vaccines (Basel) 9*, 549.

Dunlop, R.H., McEwen, S.A., Meek, A.H., Clarke, R.C., Black, W.D., Friendship, R.M. 1998. Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada. *Prev Vet Med 34*, 283-305.

Dyer, N.W., Krogh, D.F., Schaan, L.P. 2004. Pulmonary Mycoplasmosis in farmed white-tailed deer (Odocoileus virginianus). *J Wildl Dis 40*, 366-370.

ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. 2015. *EFSA J 15*, e04872.

Edwards, T.A. 2010. Control methods for bovine respiratory disease for feedlot cattle. *Vet Clin North Am Food Anim Pract 26*, 273-284.

Faucher, M., Nouvel, L.X., Dordet-Frisoni, E., Sagné, E., Baranowski, E., Hygonenq, M.C., Marenda, M.S., Tardy, F., Citti, C. 2019. Mycoplasmas under experimental antimicrobial selection: The unpredicted contribution of horizontal chromosomal transfer. *PLoS Genet 15*, e1007910.

Fertner, M., Toft, N., Martin, H.L., Boklund, A. 2016. A register-based study of the antimicrobial usage in Danish veal calves and young bulls. *Prev Vet Med 131*, 41-47.

Foster, A.P., Naylor, R.D., Howie, N.M., Nicholas, R.A.J., Ayling, R.D. 2009. *Mycoplasma bovis* and otitis in dairy calves in the United Kingdom. *Vet J 179,* 455-457.

Fox, L.K., Hancock, D.D., Mickelson, A., Britten, A. 2003. Bulk tank milk analysis: factors associated with appearance of *Mycoplasma* sp. in milk. *J Vet Med B Infect Dis Vet Public Health 50*, 235-40.

Fox, L.K. 2012. Mycoplasma mastitis. Causes, transmission, and control. *Vet Clin North Am Food Anim Pract 28,* 225-237.

Francoz, D., Buczinski, S., Apley, M. 2012. Evidence related to the use of ancillary drugs in Bovine

Respiratory Disease (anti-inflammatory and others): Are they justified or not? *Vet Clin North Am Food Anim Pract 28*, 23-38.

Francoz, D., Fecteau, G., Desrochers, A., Fortin, M. 2004. Otitis media in dairy calves: A retrospective study of 15 cases (1987 to 2002). *Can Vet J 45,* 661-666.

Francoz, D., Fortin, M., Fecteau, G., Messier, S. 2005. Determination of *Mycoplasma bovis* susceptibilities against six antimicrobial agents using the E test method. *Vet Microbiol 105,* 57-64.

Francoz, D., Buczinski, S., Belanger, A.M., Forte, G., Labrecque, O., Tremblay, D., Wellemans, V., Dubuc, J. 2015. Respiratory pathogens in Québec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. *J Vet Intern Med 29*, 381-387.

Gagea, M.I., Bateman, K.G., Shanahan, R.A., Van Dreumel, T., McEwen, B.J., Carman, S., Archambault, M., Caswell, J.L. 2006. Naturally occurring *Mycoplasma bovis*-associated pneumonia and polyarthritis in feedlot beef calves. *J Vet Diagnostic Investig 18,* 29-40.

García-Galán, A., Nouvel, L.X., Baranowski, E., Gómez-Martín, Á., Sánchez, A., Citti, C., de la Fe, C. 2020. *Mycoplasma bovis* in Spanish cattle herds: Two groups of multiresistant isolates predominate, with one remaining susceptible to fluoroquinolones. *Pathogens 9*, 545.

Gautier-Bouchardon, A. V. 2018. Antimicrobial Resistance in *Mycoplasma* spp. *Microbiol Spectr 6*.

Gautier-Bouchardon, A. V., Ferré, S., Le Grand, D., Paoli, A., Gay, E., Poumarat, F. 2014. Overall decrease in the susceptibility of *Mycoplasma bovis* to antimicrobials over the past 30 years in France. *PLoS One 9*, e87672.

Gerchman, I., Levisohn, S., Mikula, I., Lysnyansky, I. 2009. In vitro antimicrobial susceptibility of *Mycoplasma bovis* isolated in Israel from local and imported cattle. *Vet Microbiol 137,* 268-275.

Giguère, S. 2013a. Macrolides, Azalides, and Ketolides, in: Antimicrobial Therapy in Veterinary Medicine.

Giguère, S. 2013b. Lincosamides, Pleuromutilins, and Streptogramins, in: Antimicrobial Therapy in Veterinary Medicine.

Giguère, S. Dowling, P.M., 2013. Fluoroquinolones, in: Antimicrobial Therapy in Veterinary Medicine.

Gille, L., Callens, J., Supré, K., Boyen, F., Haesebrouck, F., Van Driessche, L., van Leenen, K., Deprez, P., Pardon, B. 2018a. Use of a breeding bull and absence of a calving pen as risk factors for the presence of *Mycoplasma bovis* in dairy herds. *J Dairy Sci 101*, 8284-8290.

Gille, L., Boyen, F., Van Driessche, L., Valgaeren, B., Haesebrouck, F., Deprez, P., Pardon, B. 2018b. Short communication: Effect of freezer storage time and thawing method on the recovery of *Mycoplasma bovis* from bovine colostrum. *J Dairy Sci 101,* 609-613.

Gille, L. 2018. *Mycoplasma bovis*: sources of infection, prevalence and risk factors. Ghent University. Faculty of Veterinary Medicine, Merelbeke, Belgium.

Gille, L., Evrard, J., Callens, J., Supré, K., Grégoire, F., Boyen, F., Haesebrouck, F., Deprez, P.,

Pardon, B. 2020. The presence of *Mycoplasma bovis* in colostrum. *Vet Res 51,* 54.

Gille, L., Pilo, P., Valgaeren, B.R., Van Driessche, L., Van Loo, H., Bodmer, M., Bürki, S., Boyen, F., Haesebrouck, F., Deprez, P., Pardon, B. 2016. A new predilection site of *Mycoplasma bovis:* Postsurgical seromas in beef cattle. *Vet Microbiol* 186, 67-70.

Gioia, G., Addis, M.F., Santisteban, C., Gross, B., Nydam, D.V., Sipka, A.s., Virkler, P.D., Watters, R.D., Wieland, M., Zurakowski, M.J., Moroni, P. 2021. *Mycoplasma* species isolated from bovine milk collected from US dairy herds between 2016 and 2019. *J Dairy Sci 104*, 4813 4821

Godinho, K.S., Rae, A., Windsor, G.D., Tilt, N., Rowan, T.G., Sunderland, S.J. 2005a. Efficacy of tulathromycin in the treatment of bovine respiratory disease associated with induced *Mycoplasma bovis* infections in young dairy calves. *Vet Ther 6*, 96-112.

Godinho, K.S., Wolf, R.M.L.G., Sherington, J., Rowan, T.G., Sunderland, S.J., Evans, N.A. 2005b. Efficacy of tulathromycin in the treatment and prevention of natural outbreaks of bovine respiratory disease in European cattle. *Vet Ther 6,* 122-135.

Gonzalez, R.N., Sears, P.M., Merrill, R.A., Hayes, G.L. 1992. Mastitis due to *Mycoplasma* in the state of New York during the period 1972-1990. *Cornell Vet 82,* 29-40.

González, R.N., Wilson, D.J. 2003. Mycoplasmal mastitis in dairy herds. *Vet Clin North Am Food Anim Pract 19*, 199-221.

Goodwin, S., McPherson, J.D., McCombie, W.R. 2016. Coming of age: Ten years of next-generation sequencing technologies. *Nat Rev Genet 17*, 333-351.

Gourlay, R.N., Thomas, L.H., Wyld, S.G., Smith, C.J. 1989. Effect of a new macrolide antibiotic (tilmicosin) on pneumonia experimentally induced in calves by *Mycoplasma bovis* and *Pasteurella haemolytica*. *Res Vet Sci 47,* 84-89.

Graveland, H., Wagenaar, J.A., Heesterbeek, H., Mevius, D., van Duijkeren, E., Heederik, D. 2010. Methicillin resistant *staphylococcus aureus* ST398 in veal calf farming: Human MRSA carriage related with animal antimicrobial usage and farm hygiene. *PLoS One 5*, e10990.

Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. 2005. *Am J Respir Crit Care Med 171*, 388-416.

Guo, Y., Luo, H., Guo, S., Lei, Y., Li, Y., He, S. 2020. Multi-locus sequence typing *of Mycoplasma bovis* to assess its genetic diversity from 2009 to 2018 in Ningxia Hui Autonomous Region, China. *BMC Vet Res 16,* 454.

Haapala, V., Herva, T., Härtel, H., Pitkänen, E., Mattila, J., Rautjoki, P., Pelkonen, S., Soveri, T., Simojoki, H. 2019. Comparison of Finnish meat inspection records and average daily gain for cattle herds differing in *Mycoplasma bovis* test-status. *Vet J 249*, 41-46.

Haapala, V., Pohjanvirta, T., Vähänikkilä, N., Halkilahti, J., Simonen, H., Pelkonen, S., Soveri, T., Simojoki, H., Autio, T. 2018. Semen as a source of *Mycoplasma bovis* mastitis in dairy herds. *Vet Microbiol 216*, 60-66.

Haines, D.M., Martin, K.M., Clark, E.G., Kee Jim, G., Janzen, E.D. 2001. The immunohistochemical detection of *Mycoplasma bovis* and bovine viral diarrhea virus in tissues of feedlot cattle with chronic, unresponsive respiratory disease and/or arthritis. *Can Vet J 42*, 857-860.

Haines, D.M., Moline, K.M., Sargent, R.A., Campbell, J.R., Myers, D.J., Doig, P.A. 2004. Immunohistochemical study of *Hemophilus somnus*, *Mycoplasma bovis, Mannheimia hemolytica* and bovine viral diarrhea virus in death losses due to myocarditis in feedlot cattle. *Can Vet J 45*, 231-234.

Hale, H.H., Helmboldt, C.F., Plastridge, W.N., Stula, E.F. 1962. Bovine mastitis caused by a *Mycoplasma* species. *Cornell Vet 52,* 582-591.

Hannan, P.C.T. 2000. Guidelines and recommendations for antimicrobial minimum inhibitory concentration (MIC) testing against veterinary *Mycoplasma* species. *Vet Res 31*, 373-395.

Hata, E., Harada, T., Itoh, M. 2019. Relationship between antimicrobial susceptibility and multilocus sequence type of *Mycoplasma bovis* isolates and development of a method for rapid detection of point mutations involved in decreased susceptibility to macrolides, lincosamides, tetracyclines,. *Appl Environ Microbiol 85*, e00575-19.

Hendrick, S.H., Bateman, K.G., Rosengren, L.B. 2013. The effect of antimicrobial treatment and preventive strategies on bovine respiratory disease and genetic relatedness and antimicrobial resistance of *Mycoplasma bovis* isolates in a western Canadian feedlot. *Can Vet J 54*, 1146-1156.

Hermeyer, K., Peters, M., Brügmann, M., Jacobsen, B., Hewicker-Trautwein, M. 2012. Demonstration of *Mycoplasma bovis* by immunohistochemistry and in situ hybridization in an aborted bovine fetus and neonatal calf. *J Vet Diagn Invest 42*, 364-369.

Heuvelink, A., Reugebrink, C., Mars, J. 2016. Antimicrobial susceptibility of *Mycoplasma bovis* isolates from veal calves and dairy cattle in the Netherlands. *Vet Microbiol 189*, 1-7.

Hoffmann, M., Luo, Y., Monday, S.R., Gonzalez-Escalona, N., Ottesen, A.R., Muruvanda, T., Wang, C., Kastanis, G., Keys, C., Janies, D., Senturk, I.F., Catalyurek, U. V., Wang, H., Hammack, T.S., Wolfgang, W.J., Schoonmaker-Bopp, D., Chu, A., Myers, R., Haendiges, J., Evans, P.S., Meng, J., Strain, E.A., Allard, M.W., Brown, E.W. 2016. Tracing origins of the *Salmonella* Bareilly strain causing a food-borne outbreak in the United States. *J Infect Dis 213*, 502-508.

Holmes, A.H., Moore, L.S.P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P.J., Piddock, L.J.V. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet 387,* 176-187.

Huang, R.A., Letendre, L.T., Banav, N., Fischer, J., Somerville, B. 2010. Pharmacokinetics of gamithromycin in cattle with comparison of plasma and lung tissue concentrations and plasma antibacterial activity. *J Vet Pharmacol Ther 33*, 227-237.

Huang, Z., Mao, C., Wei, Y., Gu, X., Cai, Q., Shen, X., Ding, H. 2020. Analysis of the mutant selection window and killing of *Mycoplasma hyopneumoniae* for doxycycline, tylosin, danofloxacin, tiamulin, and valnemulin. *PLoS One 15*, e0220350.

Ismail, M., El-Kattan, Y.A. 2007. Comparative pharmacokinetics of marbofloxacin in healthy and *Mannheimia haemolytica* infected calves. *Res Vet Sci 82*, 398-404.

Itoh, M., Hirano, Y., Yamakawa, K., Yasutomi, I., Kuramoto, K., Furuoka, M., Yamada, K. 2020. Combination of procedure for ultra rapid extraction (PURE) and loop-mediated isothermal amplification (LAMP) for rapid detection of mycoplasma bovis in milk. *J Vet Med Sci 82*, 875-880.

Jaillard, M., Lima, L., Tournoud, M., Mahé, P., van Belkum, A., Lacroix, V., Jacob, L. 2018. A fast and agnostic method for bacterial genome-wide association studies: Bridging the gap between k-mers and genetic events. *PloS Genet 14*, e1007758.

Janardhan, K.S., Hays, M., Dyer, N., Oberst, R.D., DeBey, B.M. 2010. *Mycoplasma bovis* outbreak in a herd of North American bison (Bison bison). *J Vet Diagn Invest 22*, 797-801.

Jarrige, N., Cazeau, G., Morignat, E., Chanteperdrix, M., Gay, E. 2017. Quantitative and qualitative analysis of antimicrobial usage in white veal calves in France. *Prev Vet Med 144*, 158-166.

Jasper, D.E., Al-Aubaidi, J.M., Fabricant, J. 1974. Epidemiologic observations on mycoplasma mastitis. *Cornell Vet 64*, 407-15.

Jaÿ, M., Ambroset, C., Tricot, A., Colin, A., Tardy, F. 2020. Population structure and antimicrobial susceptibility of *Mycoplasma ovipneumoniae* isolates in France. *Vet Microbiol 248*, 108828.

Jelinski, M., Kinnear, A., Gesy, K., Andrés-Lasheras, S., Zaheer, R., Weese, S., McAllister, T.A. 2020. Antimicrobial sensitivity testing of *Mycoplasma bovis* isolates derived from western Canadian feedlot cattle. *Microorganisms 8*, 124.

Jimbo, S., Suleman, M., Maina, T., Prysliak, T., Mulongo, M., Perez-Casal, J. 2017. Effect of *Mycoplasma bovis* on bovine neutrophils. *Vet Immunol Immunopathol 188*, 27-33.

Josten, M., Reif, M., Szekat, C., Al-Sabti, N., Roemer, T., Sparbier, K., Kostrzewa, M., Rohde, H., Sahl, H.G., Bierbaum, G. 2013. Analysis of the matrix-assisted laser desorption ionization-time of flight mass spectrum of *Staphylococcus aureus* identifies mutations that allow differentiation of the main clonal lineages. *J Clin Microbiol 51*, 1809-1817.

Justice-Allen, A., Trujillo, J., Corbett, R., Harding, R., Goodell, G., Wilson, D. 2010. Survival and replication of *Mycoplasma* species in recycled bedding sand and association with mastitis on dairy farms in Utah. *J Dairy Sci 93*, 192-202.

Kahlmeter, G., Giske, C.G., Kirn, T.J., Sharp, S.E. 2019. Point-counterpoint: Differences between the European Committee on Antimicrobial susceptibility testing and Clinical and Laboratory standards institute recommendations for reporting antimicrobial susceptibility results. *J Clin Microbiol 57,* e01129-19.

Kanda, T., Tanaka, S., Suwanruengsri, M., Sukmawinata, E., Uemura, R., Yamaguchi, R., Sueyoshi, M. 2019. Bovine Endocarditis Associated with *Mycoplasma bovis. J Comp Pathol 171*, 53-58.

Katsuda, K., Kohmoto, M., Mikami, O., Tamamura, Y., Uchida, I. 2012. Plasmid-mediated florfenicol resistance in *Mannheimia haemolytica* isolated from cattle. *Vet Microbiol 155*, 444-447.

Kauf, A.C.W., Rosenbusch, R.F., Paape, M.J., Bannerman, D.D. 2007. Innate immune response to intramammary *Mycoplasma bovis* infection. *J Dairy Sci 90*, 3336-3348.

Kehrenberg, C., Schwarz, S. 2005. Plasmid-borne florfenicol resistance in *Pasteurella multocida. J Antimicrob Chemother 55*, 773-735.

Khalil, D., Becker, C.A.M., Tardy, F. 2017. Monitoring the decrease in susceptibility to ribosomal RNAs targeting antimicrobials and its molecular basis in clinical *Mycoplasma bovis* isolates over time. *Microb Drug Resist 23*, 799-811.

Khalil, D., Becker, C.A.M., Tardy, F. 2015. Alterations in the quinolone resistance-determining regions and fluoroquinolone resistance in clinical isolates and laboratory-derived mutants of *Mycoplasma bovis*: Not all genotypes may be equal. *Appl Environ Microbiol 82*, 1060-1068.

Kinnear, A., McAllister, T.A., Zaheer, R., Waldner, M., Ruzzini, A.C., Andrés-Lasheras, S., Parker, S., Hill, J.E., Jelinski, M.D. 2020. Investigation of macrolide resistance genotypes in *Mycoplasma bovis* isolates from canadian feedlot cattle. *Pathogens 9*, 622.

Kinnear, A., Waldner, M., McAllister, T.A., Zaheer, R., Register, K., Jelinski, M. 2021. Application of four genotyping methods to *Mycoplasma bovis* isolates derived from Western Canadian feedlot cattle. *J Clin Microbiol*.

Klein, U., de Jong, A., Moyaert, H., El Garch, F., Leon, R., Richard-Mazet, A., Rose, M., Maes, D., Pridmore, A., Thomson, J.R., Ayling R.D. 2017. Antimicrobial susceptibility monitoring of *Mycoplasma hyopneumoniae* and *Mycoplasma bovis* isolated in Europe. *Vet. Microbiol. 204*, 188-193.

Klein, U., de Jong, A., Youala, M., El Garch, F., Stevenin, C., Moyaert, H., Rose, M., Catania, S., Gyuranecz, M., Pridmore, A., Ayling, R.D. 2019. New antimicrobial susceptibility data from monitoring of *Mycoplasma bovis* isolated in Europe. *Vet Microbiol 238*, 108432.

Kong, L.C., Gao, D., Jia, B.Y., Wang, Z., Gao, Y.H., Pei, Z.H., Liu, S.M., Xin, J.Q., Ma, H.X. 2016. Antimicrobial susceptibility and molecular characterization of macrolide resistance of *Mycoplasma bovis* isolates from multiple provinces in China. *J Vet Med Sci 78*, 293-296.

Köser, C.U., Holden, M.T.G., Ellington, M.J., Cartwright, E.J.P., Brown, N.M., Ogilvy-Stuart, A.L., Hsu, L.Y., Chewapreecha, C., Croucher, N.J., Harris, S.R., Sanders, M., Enright, M.C., Dougan, G., Bentley, S.D., Parkhill, J., Fraser, L.J., Betley, J.R., Schulz-Trieglaff, O.B., Smith, G.P., Peacock, S.J. 2012. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. *N Engl J Med 366*, 2267-2275.

Kronvall, G. 2010. Normalized resistance interpretation as a tool for establishing epidemiological MIC susceptibility breakpoints. *J Clin Microbiol 48*, 4445-4452.

Kronvall, G., Smith, P. 2016. Normalized resistance interpretation, the NRI method: Review of NRI disc test applications and guide to calculations. *APMIS 124*, 1023-1030.

Kuipers, A., Koops, W.J., Wemmenhove, H. 2016. Antibiotic use in dairy herds in the Netherlands from 2005 to 2012. *J Dairy Sci 99*, 1632-1648.

Kumar, A., Verma, A.K., Gangwar, N.K., Rahal, A. 2012. Isolation, characterization and antibiogram of *Mycoplasma bovis* in sheep Pneumonia. *Asian J Anim Vet Adv 7*, 149-157.

Kumar, R., Register, K., Christopher-Hennings, J., Moroni, P., Gioa, G., Garcia-Fernandez, N., Nelson, J., Jelinski, M., Lysnyansky, I., Bayles, D., Alt, D., Scaria, J. 2020. Global population genomic analysis of *Mycoplasma bovis* isolates reveals transcontinental variations and potential virulence genes. *bioRxiv*.

Kusiluka, L.J.M., Ojeniyi, B., Friis, N.F. 2000. Increasing Prevalence of *Mycoplasma bovis* in Danish *Acta Vet Scand 41*, 139-146.

Lallemand, E.A., Lacroix, M.Z., Toutain, P.L., Boullier, S., Ferran, A.A., Bousquet-Melou, A. 2016. In vitro degradation of antimicrobials during use of broth microdilution method can increase the measured minimal inhibitory and minimal bactericidal concentrations. *Front Microbiol 7*, 2051.

Lamm, C.G., Munson, L., Thurmond, M.C., Barr, B.C., George, L.W. 2004. Mycoplasma otitis in California calves. *J Vet Diagn Invest 16*, 397-402.

Lange, C., Schubert, S., Jung, J., Kostrzewa, M., Sparbier, K. 2014. Quantitative matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid resistance detection. *J Clin Microbiol 52*, 4155-4162.

Lava, M., Schüpbach-Regula, G., Steiner, A., Meylan, M. 2016. Antimicrobial drug use and risk factors associated with treatment incidence and mortality in Swiss veal calves reared under improved welfare conditions. *Prev Vet Med 126*, 121-130.

Le Roy, C., Bébéar, C., Pereyre, S. 2020. Clinical evaluation of three commercial PCR assays for the detection of macrolide resistance in mycoplasma genitalium. *J Clin Microbiol 58*, e01478-19.

Leclercq, R. 2002. Mechanisms of resistance to macrolides and lincosamides: Nature of the resistance elements and their clinical implications. *Clin Infect Dis 34*, 482-492.

Ledger, L., Eidt, J., Cai, H.Y. 2020. Identification of antimicrobial resistance-associated genes through whole genome sequencing of *Mycoplasma bovis* isolates with different antimicrobial resistances. *Pathogens 9*, 588.

Leekitcharoenphon, P., Nielsen, E.M., Kaas, R.S., Lund, O., Aarestrup, F.M. 2014. Evaluation of whole genome sequencing for outbreak detection of *Salmonella* Enterica. *PLoS One 9*, e87991.

Lees, P., Shojaee Aliabadi, F. 2002. Rational dosing of antimicrobial drugs: Animals versus humans. *Int J Antimicrob Agents 19*, 269-284.

Leslie, K.E., Petersson-Wolfe, C.S. 2012. Assessment and management of pain in dairy cows with clinical mastitis. *Vet Clin North Am Food Anim Pract 28,* 289-305.

Lévesque, S., Dufresne, P.J., Soualhine, H., Domingo, M., Bekal, S., Lefebvre, B., Tremblay, C. 2015. A side by side comparison of Bruker Biotyper and VITEK MS: utility of MALDI-TOF MS technology for microorganism identification in a public health reference laboratory. *PLoS One 10*, e0144878.

Levisohn, S., Garazi, S., Gerchman, I., Brenner, J. 2004. Diagnosis of a mixed mycoplasma infection

associated with a severe outbreak of bovine pinkeye in young calves. *J Vet Diagn Invest 16*, 579-581.

Lhermie, G., La Ragione, R.M., Weese, J.S., Olsen, J.E., Christensen, J.P., Guardabassi, L. 2020. Indications for the use of highest priority critically important antimicrobials in the veterinary sector. *J Antimicrob Chemother 75*, 1671-1680.

Li, S.L., Sun, H.M., Zhu, B.L., Liu, F., Zhao, H.Q. 2017. Whole genome analysis reveals new insights into macrolide resistance in *Mycoplasma pneumoniae*. *Biomed Environ Sci 30*, 343-350.

Liu, Y., Xu, S., Li, M., Zhou, M., Huo, W., Gao, J., Liu, G., Kastelic, J.P., Han, B. 2020. Molecular characteristics and antibiotic susceptibility profiles of *Mycoplasma bovis* associated with mastitis on dairy farms in China. *Prev Vet Med 182*, 105106.

Lluch-Senar, M., Luong, K., Lloréns-Rico, V., Delgado, J., Fang, G., Spittle, K., Clark, T.A., Schadt, E., Turner, S.W., Korlach, J., Serrano, L. 2013. Comprehensive Methylome Characterization of *Mycoplasma genitalium* and *Mycoplasma pneumoniae* at Single-Base Resolution. *PLoS Genet 9*, e1003191.

Lysnyansky, I., Ayling, R.D. 2016. *Mycoplasma bovi*s: Mechanisms of resistance and trends in antimicrobial susceptibility. *Front Microbiol 27*, 595.

Lysnyansky, I., Freed, M., Rosales, R.S., Mikula, I., Khateb, N., Gerchman, I., van Straten, M., Levisohn, S. 2016. An overview of *Mycoplasma bovis* mastitis in Israel (2004-2014). *Vet J 207*, 180-183.

Lysnyansky, I., Mikula, I., Gerchman, I., Levisohn, S. 2009. Rapid detection of a point mutation in the parC gene associated with decreased susceptibility to fluoroquinolones in *Mycoplasma bovis*. *Antimicrob Agents Chemother 53*, 4911-4914.

Lysnyansky, I., Sachse, K., Rosenbusch, R., Levisohn, S., Yogev, D. 1999. The vsp locus of *Mycoplasma bovis*: Gene organization and structural features. *J Bacteriol 181*, 5734-41.

Madoff, S., Pixley, B.Q., DelGiudice, R.A., Moellering, R.C. 1979. Isolation of *Mycoplasma bovis* from a patient with systemic illness. *J Clin Microbiol 9*, 709-711.

Maeda, T., Shibahara, T., Kimura, K., Wada, Y., Sato, K., Imada, Y., Ishikawa, Y., Kadota, K. 2003. *Mycoplasma bovis*-associated suppurative otitis media and pneumonia in bull calves. *J Comp Pathol 129*, 100-110.

Maiden, M.C.J., Van Rensburg, M.J.J., Bray, J.E., Earle, S.G., Ford, S.A., Jolley, K.A., McCarthy, N.D. 2013. MLST revisited: The gene-by-gene approach to bacterial genomics. *Nat Rev Microbiol 11*, 728-736.

Malmberg, J.L., O'Toole, D., Creekmore, T., Peckham, E., Killion, H., Vance, M., Ashley, R., Johnson, M., Anderson, C., Vasquez, M., Sandidge, D., Mildenberger, J., Hull, N., Bradway, D., Cornish, T., Register, K.B., Sondgeroth, K.S. 2020. *Mycoplasma bovis* Infections in Free-Ranging Pronghorn, Wyoming, USA. *Emerg Infect Dis 26*, 2807-2814.

Martiny, D., Visscher, A., Catry, B., Chatellier, S., Vandenberg, O. 2013. Optimization of

Campylobacter growth conditions for further identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). *J Microbio Methods 94*, 221-223.

Masukagami, Y., De Souza, D.P., Dayalan, S., Bowen, C., O'Callaghan, S., Kouremenos, K., Nijagal, B., Tull, D., Tivendale, K.A., Markham, P.F., McConville, M.J., Browning, G.F., Sansom, F.M. 2017. Comparative metabolomics of *Mycoplasma bovis* and *Mycoplasma gallisepticum* reveals fundamental differences in active metabolic pathways and suggests novel gene annotations. *mSystems 2*, e00055-17. .

Maunsell, F., Brown, M.B., Powe, J., Ivey, J., Woolard, M., Love, W., Simecka, J.W. 2012. Oral inoculation of young dairy calves with *Mycoplasma bovis* results in colonization of tonsils, development of otitis media and local immunity. *PLoS One 7*, e44523.

Maunsell, F.P., Donovan, G.A. 2009. *Mycoplasma bovis* infections in young calves. *Vet Clin North Am Food Anim Pract 25*, 139-177.

Maunsell, F.P., Donovan, G.A., Risco, C., Brown, M.B. 2009. Field evaluation of a *Mycoplasma bovis* bacterin in young dairy calves. *Vaccine 27*, 2781-2788.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle. *J Vet Intern Med 25*, 772-783.

Maurin, M. 2012. Real-time PCR as a diagnostic tool for bacterial diseases. *Expert Rev Mol Diagn 12*, 731-754.

McAuliffe, L., Ellis, R.J., Miles, K., Ayling, R.D., Nicholas, R.A.J. 2006. Biofilm formation by *Mycoplasma* species and its role in environmental persistence and survival. *Microbiology 152,* 913-922.

McAuliffe, L., Kokotovic, B., Ayling, R.D., Nicholas, R.A.J. 2004. Molecular epiderniological analysis of *Mycoplasma bovis* isolates from the United Kingdom shows two genetically distinct clusters. *J Clin Microbiol 42*, 4556-4556.

McCabe, M.S., Cormican, P., Johnston, D., Earley, B. 2018. Simultaneous detection of DNA and RNA virus species involved in bovine respiratory disease by PCR-free rapid tagmentation-based library preparation and MinION nanopore sequencing. *bioRxiv*.

Menghwar, H., He, C., Zhang, H., Zhao, G., Zhu, X., Khan, F.A., Faisal, M., Rasheed, M.A., Zubair, M., Memon, A.M., Ridley, A., Robertson, I.D., Chen, Y., Guo, A. 2017. Genotype distribution of Chinese *Mycoplasma bovis* isolates and their evolutionary relationship to strains from other countries. *Microb Pathog 111*, 108-117.

Miles, K., McAuliffe, L., Persson, A., Ayling, R.D., Nicholas, R.A.J. 2005. Insertion sequence profiling of UK *Mycoplasma bovis* field isolates. *Vet Microbiol 107*, 301-306.

Miles, R.J. 1992. Catabolism in mollicutes. *J Gen Microbiol 138*, 1773-1783.

Mulongo, M., Prysliak, T., Perez-Casal, J. 2013. Vaccination of feedlot cattle with extracts and membrane fractions from two *Mycoplasma bovis* isolates results in strong humoral immune

responses but does not protect against an experimental challenge. *Vaccine 31*, 1406-1412.

Murai, K., Higuchi, H. 2019. Prevalence and risk factors of *Mycoplasma bovis* infection in dairy farms in northern Japan. *Res Vet Sci 123*, 29-31.

National Coordinating Council for Medication Error Reporting and Prevention (NCCMERP), 2021. About medication errors: what is a medication error? Available at: https://www.nccmerp.org/about-medication-errors (last accessed March 3rd, 2021).

Nicholas, R.A.J., Ayling, R.D., Stipkovits, L.P. 2002. An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis*: clinical, cultural, serological and pathological findings. *Vaccine 20*, 3569-3575.

Nicholas, R., Ayling, R., McAuliffe, L. 2008. Mycoplasma diseases of ruminants, Mycoplasma Diseases of Ruminants.

Nicholas, R. Ayling, R., McAuliffe, L. 2008. Mycoplasma Diseases of Ruminants, 1st ed.; CABI Publishing: Oxford, UK, 2008

Nicholas, R., Baker, S., Ayling, R., Stipkovits, L. 2000. Mycoplasma infections in growing cattle. *Cattle Pract 8*, 115-118.

Nicholas, R.A.J., Ayling, R.D., McAuliffe, L. 2009. Vaccines for Mycoplasma diseases in animals and man. *J Comp Pathol 140*, 85-96.

Nicholas, R.A., Fox, L.K., Lysnyansky, I. 2016. *Mycoplasma* mastitis in cattle: to cull or not to cull. *Vet J 216,* 142-147.

Nicholas, R.A.J., Loria, G.R., Catania, S., Piccinini, R. 2019. Evaluation of a saponised Mycoplasma *bovis* vaccine: some field observations. *Austin J Vet Sci and Anim Husb*. 6, 1060.

Nowakowski, M.A., Inskeep, P.B., Risk, J.E., Skogerboe, T.L., Benchaoui, H.A., Meinert, T.R., Sherington, J., Sunderland, S.J. 2004. Pharmacokinetics and lung tissue concentrations of tulathromycin, a new triamilide antibiotic, in cattle. *Vet Ther 5,* 60-74.

Nunoya, T., Omori, T., Tomioka, H., Umeda, F., Suzuki, T., Uetsuka, K. 2020. Intracellular Localization of *Mycoplasma bovis* in the bronchiolar epithelium of experimentally infected calves. *J Comp Pathol 176,* 14-18.

OIE (World Organization for Animal Health), 2019. OIE List of antimicrobial agents of veterinary importance. 2019. https://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_OIE_List_antimicrobials_July2019.pdf

Oliveira, T.E.S., Pelaquim, I.F., Flores, E.F., Massi, R.P., Valdiviezo, M.J.J., Pretto-Giordano, L.G., Alfieri, A.A., Saut, J.P.E., Headley, S.A. 2020. *Mycoplasma bovis* and viral agents associated with the development of bovine respiratory disease in adult dairy cows. *Transbound Emerg Dis 67*, 82-93.

Omaleki, L., Blackall, P.J., Cuddihy, T., Beatson, S.A., Forde, B.M., Turni, C. 2020. Using genomics to understand inter-and intra-outbreak diversity of *Pasteurella multocida* isolates associated with

fowl cholera in meat chickens. *Microb Genom 6*, e000346.

Ongor, H., Kalin, R., Karahan, M., Cetinkaya, B., McAuliffe, L., Nicholas, R.A.J. 2008. Isolation of *Mycoplasma bovis* from broiler chickens in Turkey. *Avian Pathol 37*, 587-588.

Otake, S., Dee, S., Corzo, C., Oliveira, S., Deen, J. 2010. Long-distance airborne transport of infectious PRRSV and *Mycoplasma hyopneumoniae* from a swine population infected with multiple viral variants. *Vet Microbiol 145*, 198-208.

Otter, A., Wright, T., Leonard, D., Richardson, M., Ayling, R. 2015. *Mycoplasma bovis* mastitis in dry dairy cows. *Vet Rec 177*, 601-602.

Owen, J.R., Noyes, N., Young, A.E., Prince, D.J., Blanchard, P.C., Lehenbauer, T.W., Aly, S.S., Davis, J.H., O'Rourke, S.M., Abdo, Z., Belk, K., Miller, M.R., Morley, P., Van Eenennaam, A.L. 2017. Whole-genome sequencing and concordance between antimicrobial susceptibility genotypes and phenotypes of bacterial isolates associated with bovine respiratory disease. *G3 Genes 7*, 3059-3071.

Oxford Nanopore technologies (ONT), 2021. https://nanoporetech.com/accuracy, 23 May 2021.

Pansri, P., Katholm, J, Krogh, K.M., Aagaar, A.K., Schmidt, L.M.B., Kudirkiene, E., Larsen, L.E., Olsen, J.E. 2020. Evaluation of novel multiplex qPCR assays for diagnosis of pathogens associated with the bovine respiratory disease complex. *Vet Jl 256*, 105425.

Pardon, B., Buczinski, S. 2020. Bovine respiratory disease diagnosis: what progress has been made in infectious diagnosis? *Vet Clin North Am Food Anim Pract 36*, 425-444.

Pardon, B., Callens, J., Maris, J., Allais, L., Van Praet, W., Deprez, P., Ribbens, S. 2020. Pathogen-specific risk factors in acute outbreaks of respiratory disease in calves. *J Dairy Sci 103,* 2556-2566.

Pardon, B., De Bleecker, K., Dewulf, J., Callens, J., Boyen, F., Catry, B., Deprez, P. 2011. Prevalence of respiratory pathogens in diseased, non-vaccinated, routinely medicated veal calves. *Vet Rec 169,* 278.

Pardon, B., De Bleecker, K., Hostens, M., Callens, J., Dewulf, J., Deprez, P. 2012. Longitudinal study on morbidity and mortality in white veal calves in Belgium. *BMC Vet Res 8*, 26.

Pardon, B., Hostens, M., Duchateau, L., Dewulf, J., De Bleecker, K., Deprez, P. 2013. Impact of respiratory disease, diarrhea, otitis and arthritis on mortality and carcass traits in white veal calves. *BMC Vet Res 9*, 79.

Pardon, B. 2019. Rationalizing Antimicrobial Treatment for Respiratory Infections : Steps to Take Today and Tomorrow. XXIV Congreso Internacional ANEMBE De Medicina Bovina. Asociación Nacional de Especialistas en Medicina Bovina de España (ANEMBE), 252–255.

Parker, A. M., House, J.K., Hazelton, M.S., Bosward, K.L., Mohler, V.L., Maunsell, F.P., Sheehy, P.A. 2016. Milk acidification to control the growth of *Mycoplasma bovis* and *Salmonella Dublin* in contaminated milk. *J Dairy Sci 99*, 9875-9884.

Parker, A. M., House, J.K., Hazelton, M.S., Bosward, K.L., Morton, J.M., Sheehy, P.A. 2017. Bulk

tank milk antibody ELISA as a biosecurity tool for detecting dairy herds with past exposure to *Mycoplasma bovis*. *J Dairy Sci 100*, 8296-8309.

Parker, Alysia M., House, J.K., Hazelton, M.S., Bosward, K.L., Sheehy, P.A. 2017. Comparison of culture and a multiplex probe PCR for identifying *Mycoplasma* species in bovine milk, semen and swab samples. *PLoS One 12*, e0173422.

Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House, J.K. 2018. A review of mycoplasma diagnostics in cattle. *J Vet Intern Med 32*, 1241-1252.

Parker, Alysia M., Shukla, A., House, J.K., Hazelton, M.S., Bosward, K.L., Kokotovic, B., Sheehy, P.A. 2016. Genetic characterization of Australian *Mycoplasma bovis* isolates through whole genome sequencing analysis. *Vet Microbiol 196*, 118-125.

Passchyn, P., Piepers, S., De Meulemeester, L., Boyen, F., Haesebrouck, F., De Vliegher, S. 2012. Between-herd prevalence of *Mycoplasma bovis* in bulk milk in Flanders, Belgium. *Res Vet Sci 92*, 219-220.

Patel, P.A., Ledeboer, N.A., Ginocchio, C.C., Condon, S., Bouchard, S., Qin, P., Karchmer, T., Peterson, L.R. 2011. Performance of the BD GeneOhm MRSA achromopeptidase assay for real-time PCR detection of methicillin-resistant *Staphylococcus aureus* in nasal specimens. *J Clin Microbiol 49*, 2266-2268.

Pavlovic, M., Huber, I., Konrad, R., Busch, U. 2013. Application of MALDI-TOF MS for the Identification of Food Borne Bacteria. *Open Microbiol. J.* doi:10.2174/1874285801307010135

Pereyre, S., Goret, J., Bébéar, C. 2016. *Mycoplasma pneumoniae*: Current knowledge on macrolide resistance and treatment. *Front Microbiol 7*, 974.

Pereyre, S., Tardy, F., Renaudin, H., Cauvin, E., Del Prá Netto Machado, L., Tricot, A., Benoit, F., Treilles, M., Bébéar, C. 2013. Identification and subtyping of clinically relevant human and ruminant mycoplasmas by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol 51*, 3314-3323.

Perez-Casal, J., Prysliak, T., Maina, T., Suleman, M., Jimbo, S. 2017. Status of the development of a vaccine against *Mycoplasma bovis. Vaccine 35,* 2902-2907.

Perez-Casal, J. 2020. Pathogenesis and Virulence of *Mycoplasma bovis*. *Vet Clin Food Anim 36*, 269-278.

Pérez, R., Palma, C., Drápela, C., Sepulveda, M., Espinoza, A., Peñailillo, A.K. 2015. Pharmacokinetics of florfenicol after intravenous administration in *Escherichia coli* lipopolysaccharide-induced endotoxaemic sheep. *J Vet Pharmacol Ther 38*, 144-149.

Petersen, M.B., Pedersen, J., Holm, D.L., Denwood, M., Nielsen, L.R. 2018a. A longitudinal observational study of the dynamics of *Mycoplasma bovis* antibodies in naturally exposed and diseased dairy cows. *J Dairy Sci 101*, 7383-7396.

Petersen, M.B., Wawegama, N.K., Denwood, M., Markham, P.F., Browning, G.F., Nielsen, L.R. 2018b. *Mycoplasma bovis* antibody dynamics in naturally exposed dairy calves according to two

diagnostic tests. *BMC Vet Res 14*, 258.

Petersen, M.B., Pedersen, L., Pedersen, L.M., Nielsen, L.R. 2020. Field experience of antibody testing against *Mycoplasma bovis* in adult cows in commercial Danish dairy cattle herds. *Pathogens 9*, 637.

Peuchant, O., Ménard, A., Renaudin, H., Morozumi, M., Ubukata, K., Bébéar, C.M., Pereyre, S. 2009. Increased macrolide resistance of *Mycoplasma pneumoniae* in France directly detected in clinical specimens by real-time PCR and melting curve analysis. *J Antimicrob Chemother 64*, 52-58.

Piccinini, R., Gosney, F., Snel, G.G.M., Vittorio Luini, M., Nicholas, R.A.J. 2015. Environmental survival of *Mycoplasma bovis* on a white veal farm. *Vet Rec Case Rep 3*, 3000207.

Pfützner, H. 1984. Tenazität von *Mycoplasma bovis. Zentralblatt Bakteriol Mikrobiol Hyg A 258*, 38-41.

Pfutzner, F.L. 1990. Epizootiology of the *Mycoplasma bovis* Infection of Cattle. In: Stanek, G., Cassell, G.H., Tully, J.G. and Whitcomb, R.F., Eds., Recent Advances in Mycoplasmology, Gustav Fisher Verlag, Stuttgart, 394-399.

Pfützner, H., Sachse, K. 1996. *Mycoplasma bovis* as an agent of mastitis, pneumonia, arthritis and genital disorders in cattle. *Rev Sci Tech 15,* 1477-1494.

Pinho, L., Thompson, G., Machado, M., Carvalheira, J. 2013. Management practices associated with the bulk tank milk prevalence of *Mycoplasma* spp. in dairy herds in Northwestern Portugal. *Prev Vet Med 108*, 21-27.

Pinho, L., Thompson, G., Rosenbusch, R., Carvalheira, J. 2012. Genotyping of *Mycoplasma bovis* isolates using multiple-locus variable-number tandem-repeat analysis. *J Microbiol Methods 88*, 377-385.

Poumarat, F., Le Grand, D., Philippe, S., Calavas, D., Schelcher, F., Cabanié, P., Tessier, P., Navetat, H. 2001. Efficacy of spectinomycin against *Mycoplasma bovis* induced pneumonia in conventionally reared calves. *Vet Microbiol 80*, 23-35.

Poveda, J.B., Nicholas, R. 1998. Serological identification of mycoplasmas by growth and metabolic inhibition tests. *Methods Mol. Biol.* doi:10.1385/0-89603-525-5:105

Prysliak, T., Perez-Casal, J. 2016. Immune responses to *Mycoplasma bovis* proteins formulated with different adjuvants. *Can J Microbiol 62*, 492-504.

Prysliak, T., van der Merwe, J., Perez-Casal, J. 2013. Vaccination with recombinant *Mycoplasma bovis* GAPDH results in a strong humoral immune response but does not protect feedlot cattle from an experimental challenge with *M. bovis*. *Microb Pathog 55*, 1-8.

Public Health England. 2020. Investigation of novel SARS-CoV-2 variant Variant of Concern 202012/01 Technical briefing 2. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/959361/Technical_Briefing_VOC202012-2_Briefing_2.pdf

Puchalski, A., Urban-Chmiel, R., Dec, M., Stęgierska, D., Wernicki, A. 2016. The use of MALDI-

TOF mass spectrometry for rapid identification of *Mannheimia haemolytica*. *J Vet Med Sci 78*, 1339-1342.

Punyapornwithaya , V., Fox, L.K., Hancock, D.D., Gay, J.M., Wenz, J.R., Alldredge, J.R. 2011. Incidence and transmission of *Mycoplasma bovis* mastitis in Holstein dairy cows in a hospital pen: A case study. *Prev Vet Med 98*, 74-8.

Radaelli, E., Luini, M., Loria, G.R., Nicholas, R.A.J., Scanziani, E. 2008. Bacteriological, serological, pathological and immunohistochemical studies of *Mycoplasma bovis* respiratory infection in veal calves and adult cattle at slaughter. *Res Vet Sci 85*, 282-290.

Raherison, S., Gonzalez, P., Renaudin, H., Charron, A., Bébéar, C., Bébéar, C.M. 2002. Evidence of active efflux in resistance to ciprofloxacin and to ethidium bromide by *Mycoplasma hominis*. *Antimicrob Agents Chemother 46,* 672-679.

Randall, L.P., Lemma, F., Koylass, M., Rogers, J., Ayling, R.D., Worth, D., Klita, M., Steventon, A., Line, K., Wragg, P., Muchowski, J., Kostrzewa, M., Whatmore, A.M. 2015. Evaluation of MALDI-ToF as a method for the identification of bacteria in the veterinary diagnostic laboratory. *Res Vet Sci 101*, 42-49.

Rang, F.J., Kloosterman, W.P., de Ridder, J. 2018. From squiggle to basepair: Computational approaches for improving nanopore sequencing read accuracy. *Genome Biol 19*, 90.

Razin S. 1978. The mycoplasmas. *Microbiological reviews*, *42*, 414–470.

Razin, A., Razin, S. 1980. Methylated bases in mycoplasmal DNA. *Nucleic Acids Res 8*, 1383-1390.

Razin, S. 1996. Chapter 37: Mycoplasmas. Med. Microbiol.

Razin, S., Hayflick, L. 2010. Highlights of mycoplasma research - An historical perspective. *Biologicals 38*, 183-190.

Redgrave, L.S., Sutton, S.B., Webber, M.A., Piddock, L.J.V. 2014. Fluoroquinolone resistance: Mechanisms, impact on bacteria, and role in evolutionary success. *Trends Microbiol 22*, 438-445.

Register, K.B., Jelinski, M.D., Waldner, M., Boatwright, W.D., Anderson, T.K., Hunter, D.L., Hamilton, R.G., Burrage, P., Shury, T., Bildfell, R., Wolff, P.L., Miskimins, D., Derscheid, R.J., Woodbury, M.R. 2019. Comparison of multilocus sequence types found among North American isolates of *Mycoplasma bovis* from cattle, bison, and deer, 2007–2017. *J Vet Diagn Invest 31*, 899-904.

Register, K.B., Lysnyansky, I., Jelinski, M.D., Boatwright, W.D., Waldner, M., Bayles, D.O., Pilo, P., Alt, D.P. 2020. Comparison of two multilocus sequence typing schemes for *Mycoplasma bovis* and revision of the PubMLST reference method. *J Clin Microbiol 58*, e00283-20.

Register, K.B., Thole, L., Rosenbush, R.F., Minion, F.C. 2015. Multilocus sequence typing of *Mycoplasma bovis* reveals host-specific genotypes in cattle versus bison. *Vet Microbiol 175,* 92-98.

Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on

veterinary medicinal products and repealing Directive 2001/82/EC. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32019R0006&qid=1617086109751

Rocha, E., Sirand-Pugnet, P., Blanchard, A. 2005. Genome analysis: recombination, repair. In: Blanchard A, Browning G, editors. Mycoplasmas: Molecular Biology Pathogenicity and Strategies for Control. Poole, UK: Horizon Bioscience. 31–73.

Rodriguez, F., Bryson, D.G., Ball, H.J., Forster, F. 1996. Pathological and immunohistochemical studies of natural and experimental *Mycoplasma bovis* pneumonia in calves. *J Comp Pathol 115*, 151-162.

Rodríguez, F., Sarradell, J., Poveda, J.B., Ball, H.J., Fernández, A. 2000. Immunohistochemical characterization of lung lesions induced experimentally by *Mycoplasma agalactiae* and *Mycoplasma bovis* in goats. *J Comp Pathol 123,* 285-293.

Rosales, R.S., Churchward, C.P., Schnee, C., Sachse, K., Lysnyansky, I., Catania, S., Iob, L., Ayling, R.D., Nicholas, R.A.J. 2015. Global multilocus sequence typing analysis of *Mycoplasma bovis* isolates reveals two main population clusters. *J Clin Microbiol 53*, 789-794.

Rosenbusch. 1994. Chapter 1. Biology and taxonomy of the Mycoplasmas. Mycoplasmosis in animals: laboratory diagnosis 1st edition 1994.

Rosenbusch, R.F. 1998. Test of an inactivated vaccine against *Mycoplasma bovis* respiratory disease by transthoracic challenge with an abscessing strain Abstracts of the 12th International Organisation of Mycoplasmology conference, Sydney, Australia, 185.

Rosenbusch, R.F., Kinyon, J.M., Apley, M., Funk, N.D., Smith, S., Hoffman, L.J. 2005. In vitro antimicrobial inhibition profiles of *Mycoplasma bovis* isolates recovered from various regions of the United States from 2002 to 2003. *J Vet Diagn Invest 17*, 436-441.

Rosengarten, R., Citti, C. 1999. The role of ruminant mycoplasmas in systemic infection. In: Stipkovits, L., Rosengarten, R., Frey, J. Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics. Vol 3. Brussels, Belgium: European Commission, 14-17.

Rottem, S. 2003. Interaction of mycoplasmas with host cells. *Physiol Rev 83*, 417-432.

Sabat, A.J., Budimir, A., Nashev, D., Sá-Leão, R., van Dijl, J.M., Laurent, F., Grundmann, H., Friedrich, A.W. 2013. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Euro Surveill 18*, 20380.

Sachse, K., Helbig, J.H., Lysnyansky, I., Grajetzki, C., Müller, W., Jacobs, E., Yogev, D. 2000. Epitope mapping of immunogenic and adhesive structures in repetitive domains of *Mycoplasma bovis* variable surface lipoproteins. *Infect Immun 68*, 680-687.

Sachse, K., Salam, H.S.H., Diller, R., Schubert, E., Hoffmann, B., Hotzel, H. 2010. Use of a novel real-time PCR technique to monitor and quantitate *Mycoplasma bovis* infection in cattle herds with mastitis and respiratory disease. *Vet J 186*, 299-303.

Sánchez-Vargas, F.M., Gómez-Duarte, O.G. 2008. *Mycoplasma pneumonia* – an emerging extra – pulmonary pathogen. *Clin Microbiol Infect 14*, 105-117.

Sato, T., Higuchi, H., Yokota, S.I., Tamura, Y. 2017. *Mycoplasma bovis* isolates from dairy calves in Japan have less susceptibility than a reference strain to all approved macrolides associated with a point mutation (G748A) combined with multiple species-specific nucleotide alterations in 23S rRNA. *Microbiol Immunol 61*, 215-224.

Sato, T., Okubo, T., Usui, M., Higuchi, H., Tamura, Y. 2013. Amino acid substitutions in gyrA and parC are associated with fluoroquinolone resistance in *Mycoplasma bovis* isolates from Japanese dairy calves. *J Vet Med Sci 75*, 1063-1065.

Sauer, S., Freiwald, A., Maier, T., Kube, M., Reinhardt, R., Kostrzewa, M., Geider, K. 2008. Classification and identification of bacteria by mass spectrometry and computational analysis. *PLoS One 3*, e2843.

Sauer, S., Kliem, M. 2010. Mass spectrometry tools for the classification and identification of bacteria. *Nat Rev Microbiol 8*, 74-82.

Schibrowski, M.L., Gibson, J.S., Hay, K.E., Mahony, T.J., Barnes, T.S. 2018. *Mycoplasma bovis* and bovine respiratory disease: A risk factor study in Australian feeder cattle. *Prev Vet Med 157*, 152-161.

Schönecker, L., Schnyder, P., Overesch, G., Schüpbach-Regula, G., Meylan, M. 2019. Associations between antimicrobial treatment modalities and antimicrobial susceptibility in *Pasteurellaceae* and *E. coli* isolated from veal calves under field conditions. *Vet Microbiol 236*, 108363.

Schwarz, S., Silley, P., Simjee, S., Woodford, N., van duijkeren, E., Johnson, A.P., Gaastra, W. 2010. Editorial: Assessing the antimicrobial susceptibility of bacteria obtained from animals. *J Antimicrob Chemother 65*, 601-604.

Shahriar, F.M., Clark, E.G., Janzen, E., West, K., Wobeser, G. 2002. Coinfection with bovine viral diarrhea virus and mycoplasma bovis in feedlot cattle with chronic pneumonia. *Can Vet J 43*, 863-868.

Shi, F., Zhao, Y., Sun, Y., Chen, C., 2020. Development and application of a colloidal carbon test strip for the detection of antibodies against *Mycoplasma bovis*. *World J Microbiol Biotechnol 36*, 157.

Silley, P. 2012. Susceptibility testing methods, resistance and breakpoints: What do these terms really mean? *Rev Sci Tech 31*, 33-41.

Siugzdaite, J., Gabinaitiene, A., Kerziene, S., 2012. Susceptibility of *Mycoplasma bovis* field isolates to antimicrobial agents. *Vet Med 57*, 575-582.

Slatko, B.E., Gardner, A.F., Ausubel, F.M. 2018. Overview of Next-Generation Sequencing Technologies. *Curr Protoc Mol Biol 122*, e59.

Sloan, G., Wang G., Cheng, K. 2017. Traditional approaches versus mass spectrometry in bacterial identification and typing. *Clin Chim Acta 473*, 180-185.

Soehnlen, Marty K., Aydin, A., Lengerich, E.J., Houser, B.A., Fenton, G.D., Lysczek, H.R., Burns, C.M., Byler, L.I., Hattel, A.L., Wolfgang, D.R., Jayarao, B.M. 2011. Blinded, controlled field trial of two commercially available *Mycoplasma bovis* bacterin vaccines in veal calves. *Vaccine*

29, 5347-5354.

Soehnlen, M.K., Aydin, A., Murthy, K.S., Lengerich, E.J., Hattel, A.L., Houser, B.A., Fenton, G.D., Lysczek, H.R., Burns, C.M., Townsend, A.M., Brooks, J.W., Wolfgang, D.R., Jayarao, B.M. 2012. Epidemiology of *Mycoplasma bovis* in Pennsylvania veal calves. *J Dairy Sci 95*, 247-254.

Soehnlen, M. K., Kariyawasam, S., Lumadue, J.A., Pierre, T.A., Wolfgang, D.R., Jayarao, B.M. 2011. Molecular epidemiological analysis of *Mycoplasma bovis* isolates from the Pennsylvania Animal Diagnostic Laboratory showing genetic diversity. *J Dairy Sci 94*, 1893-1899.

Sparbier, K., Schubert, S., Kostrzewa, M. 2016. MBT-ASTRA: A suitable tool for fast antibiotic susceptibility testing? *Methods 104*, 48-54.

Spergser, J., Hess, C., Loncaric, I., Ramírez, A.S. 2019. Matrix-assisted laser desorption ionization-time of flight mass spectrometry is a superior diagnostic tool for the identification and differentiation of mycoplasmas isolated from animals. *J Clin Microbiol 57,* e00316-19.

Spergser, J., Macher, K., Kargl, M., Lysnyansky, I., Rosengarten, R. 2013. Emergence, re-emergence, spread and host species crossing of *Mycoplasma bovis* in the Austrian Alps caused by a single endemic strain. *Vet Microbiol 164*, 299-306.

Stanford, K., Zaheer, R., Klima, C., McAllister, T., Peters, D., Niu, Y.D., Ralston, B. 2020. Antimicrobial resistance in members of the bacterial bovine respiratory disease complex isolated from lung tissue of cattle mortalities managed with or without the use of antimicrobials. *Microorganisms* 8, 288.

Stipkovits, L., Rády, M., Glávits, R. 1993. Mycoplasmal arthritis and meningitis in calves. *Acta Vet Hung 41*, 73-88.

Stipkovits, L., Ripley, P., Varga, J., Pálfi, V. 2000. Clinical study of the disease of calves associated with *Mycoplasma bovis* infection. *Acta Vet Hung 48*, 387-395.

Stipkovits, L., Ripley, P.H., Tenk, M., Glávits, R., Molnár, T., Fodor, L. 2005. The efficacy of valnemulin (Econor®) in the control of disease caused by experimental infection of calves with *Mycoplasma bovis. Res Vet Sci 78*, 207-215.

Stipkovits, L., Ripley, P.H., Varga, J., Palfi, V. 2001. Use of valnemulin in the control of *Mycoplasma bovis* infection under field conditions. *Vet Rec 148*, 399-402.

Suleman, M., Prysliak, T., Clarke, K., Burrage, P., Windeyer, C., Perez-Casal, J. 2016. *Mycoplasma bovis* isolates recovered from cattle and bison (Bison bison) show differential in vitro effects on PBMC proliferation, alveolar macrophage apoptosis and invasion of epithelial and immune cells. *Vet Microbiol 186*, 28-36.

Sulyok, K.M., Bekő, K., Kreizinger, Z., Wehmann, E., Jerzsele, Á., Rónai, Z., Turcsányi, I., Makrai, L., Szeredi, L., Jánosi, S., Nagy, S.Á., Gyuranecz, M. 2018. Development of molecular methods for the rapid detection of antibiotic susceptibility of *Mycoplasma bovis. Vet Microbiol 213*, 47-57.

Sulyok, K.M., Kreizinger, Z., Fekete, L., Jánosi, S., Schweitzer, N., Turcsányi, I., Makrai, L., Erdélyi,

K., Gyuranecz, M. 2014. Phylogeny of *Mycoplasma bovis* isolates from Hungary based on multi locus sequence typing and multiple-locus variable-number tandem repeat analysis. *BMC Vet Res 10*, 108.

Sulyok, K.M., Kreizinger, Z., Wehmann, E., Lysnyansky, I., Bányai, K., Marton, S., Jerzsele, A., Rónai, Z., Turcsányi, I., Makrai, L., Jánosi, S., Nagy, S.Á., Gyuranecz, M. 2017. Mutations associated with decreased susceptibility to seven antimicrobial families in field and laboratory-derived *Mycoplasma bovis* strains. *Antimicrob Agents Chemother 61*, e01983-16.

Szacawa, E., Niemczuk, K., Dudek, K., Bednarek, D., Rosales, R., Ayling, R. 2015. *Mycoplasma bovis* infections and co-infections with other *Mycoplasma* spp. with different clinical manifestations in affected cattle herds in eastern region of Poland. *Bull Vet Inst Pulawy 59*, 331-338.

Tardy, F., Aspan, A., Autio, T., Ridley, A., Tricot, A., Colin, A., Pohjanvirta, T., Smid, B., Harders, F., Lindegaard, M., Lauritsen, K.T., Lyhs, U., Wisselink, H.J., Strube, M.L. 2020. *Mycoplasma bovis* in nordic european countries: Emergence and dominance of a new clone. *Pathogens 9*, 875.

Tardy, F., Baranowski, E., Nouvel, L.X., Mick, V., Manso-Silva'n, L., Thiaucourt, F., Thébault, P., Breton, M., Sirand-Pugnet, P., Blanchard, A., Garnier, A., Gibert, P., Game, Y., Poumarat, F., Citti, C. 2012. Emergence of atypical *Mycoplasma agalactiae* strains harboring a new prophage and associated with an alpine wild ungulate mortality episode. *Appl Environ Microbiol 78*, 4659-4668.

Tardy, F., Mick, V., Dordet-Frisoni, E., Marenda, M.S., Sirand-Pugnet, P., Blanchard, A., Citti, C. 2015. Integrative conjugative elements are widespread in field isolates of *Mycoplasma* species pathogenic for ruminants. *Appl Environ Microbiol 81*, 1634-1643.

Tassew, D.D., Mechesso, A.F., Park, N.H., Song, J.B., Shur, J.W., Park, S.C. 2017. Biofilm formation and determination of minimum biofilm eradication concentration of antibiotics in *Mycoplasma hyopneumonia*e. *J Vet Med Sci 79,* 1716-1720.

TeKippe, E.M., Burnham, C.A.D. 2014. Evaluation of the Bruker Biotyper and VITEK MS MALDI-TOF MS systems for the identification of unusual and/or difficult-to-identify microorganisms isolated from clinical specimens. 2014. *Eur J Clin Microbiol Infect Dis 33*, 2163-2171.

The Netherlands Veterinary Medicines Institute (SDa). 2020. Appendix to the report Usage of Antibiotics in Agricultural Livestock in the Netherlands in 2019 Trends and benchmarking of livestock farms and veterinarians.

Theuns, S., Vanmechelen, B., Bernaert, Q., Deboutte, W., Vandenhole, M., Beller, L., Matthijnssens, J., Maes, P., Nauwynck, H.J. 2018. Nanopore sequencing as a revolutionary diagnostic tool for porcine viral enteric disease complexes identifies porcine kobuvirus as an important enteric virus. *Sci Rep 8*, 9830.

Thiry, J., González-Martín, J. V., Elvira, L., Pagot, E., Voisin, F., Lequeux, G., Weingarten, A., De Haas, V. 2014. Treatment of naturally occurring bovine respiratory disease in juvenile calves

with a single administration of a florfenicol plus flunixin meglumine formulation. *Vet Rec 174*, 430.

Thomas, C.B., Willeberg, P., Jasper, D.E. 1981. Case-control study of bovine mycoplasmal mastitis in California. *Am J Vet Res*. 42, 511-5.

Thomas, A., Ball, H., Dizier, I., Trolin, A., Bell, C., Mainil, J., Linden, A. 2002. Isolation of *Mycoplasma* species from the lower respiratory tract of healthy cattle and cattle with respiratory disease in Belgium. *Vet Rec 151*, 472-476.

Thomas, A., Linden, A., Mainil, J., Bischof, D.F., Frey, J., Vilei, E.M. 2005. *Mycoplasma bovis* shares insertion sequences with *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. *mycoides* SC: Evolutionary and developmental aspects. *FEMS Microbiol Lett 245*, 249-255.

Thomas, A., Nicolas, C., Dizier, I., Mainil, J., Linden, A. 2003. Antibiotic susceptibilities of recent isolates of *Mycoplasma bovis* in Belgium. *Vet Rec 153*, 428-431.

Thompson, C.C., Vieira, N.M., Carolina, A., Vicente, P., Thompson, F.L. 2011. Towards a genome based taxonomy of Mycoplasmas. *Infect Genet Evol 11*, 1798-1804.

Thomson, K., Rantala, M., Hautala, M., Pyörälä, S., Kaartinen, L. 2008. Cross-sectional prospective survey to study indication-based usage of antimicrobials in animals: Results of use in cattle. *BMC Vet Res 4*, 15.

Timsit, E., Arcangioli, M.A., Bareille, N., Seegers, H., Assié, S. 2012. Transmission dynamics of *Mycoplasma bovis* in newly received beef bulls at fattening operations. *J Vet Diag Invest* 24, 1172-1176.

Timsit, E. Christensen, H., Bareille, N., Seegers, H., Bisgaard, M. Assié, S. 2013. Transmission dynamics of *Mannheimia haemolytica* in newly-received beef bulls at fattening operations. *Vet Microbiol 161*, 295-304.

Toutain, P.L., Bousquet-Mélou, A., Damborg, P., Ferran, A.A., Mevius, D., Pelligand, L., Veldman, K.T., Lees, P. 2017. En Route towards European Clinical breakpoints for veterinary antimicrobial susceptibility testing: A position paper explaining the VetCAST approach. *Front Microbiol 8*, 2344.

Travisano, M. 2001. Bacterial genetics. Encyclopedia of Biodiversity. Editor-in-Chief S.A. Levin, Academic press.

Tschopp, R., Bonnemain, P., Nicolet, J., Burnens, A. 2001. Epidemiological study of risk factors associated with *Mycoplasma bovis* infections in fattening calves. *Schweiz Arch Tierheilkd 143*, 461-467.

Turnidge, J., Kahlmeter, G., Kronvall, G. 2006. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clin Microbiol Infect 12,* 418-425.

Turnidge, J., Paterson, D.L. 2007. Setting and revising antibacterial susceptibility breakpoints. *Clin Microbiol Rev 20*, 391-408.

Uemura, R., Sueyoshi, M., Nagatomo, H. 2010. Antimicrobial susceptibilities of four species of *Mycoplasma* isolated in 2008 and 2009 from cattle in Japan. *J Vet Med Sci 72*, 1661-1663.

University of Western Australia (UWA), Faculty of Medicine, Dentistry and Health Sciences. 2021. Understanding absolute and relative risk reduction. https://www.meddent.uwa.edu.au/__data/assets/pdf_file/0005/2670593/Risk_reduction_guide0.2.pdf (last accessed March 30th).

Urbaneck, D., Liebig, F., Forbrig, T., Stache, B. 2000. Erfahrungsbericht zur Anwendung bestandsspezifischer Impfstoffe gegen respiratorische Infektionen mit Beteiligung von *Mykoplasma bovis* in einem Mastrindergroßbestand. *Prakt Tierarzt 82*, 756-763.

Vähänikkilä, N., Pohjanvirta, T., Haapala, V., Simojoki, H., Soveri, T., Browning, G.F., Pelkonen, S., Wawegama, N.K., Autio, T. 2019. Characterisation of the course of *Mycoplasma bovis* infection in naturally infected dairy herds. *Vet Microbiol 231*, 107-115.

Van Belkum, A. 2007. Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). FEMS *Immunol Med Microbiol 49*, 22-27.

Van Der Merwe, J., Prysliak, T., Perez-Casal, J. 2010. Invasion of bovine peripheral blood mononuclear cells and erythrocytes by *Mycoplasma bovis*. *Infect Immun 78*, 4570-4578.

Van Driessche, L., Valgaeren, B.R., Gille, L., Boyen, F., Ducatelle, R., Haesebrouck, F., Deprez, P., Pardon, B. 2017. A deep nasopharyngeal swab versus nonendoscopic bronchoalveolar lavage for isolation of bacterial pathogens from preweaned calves with respiratory disease. *J Vet Intern Med 31*, 946-953.

Van Driessche, L., Bokma, J., Gille, L., Ceyssens, P.J., Sparbier, K., Haesebrouck, F., Deprez, P., Boyen, F., Pardon, B. 2018. Rapid detection of tetracycline resistance in bovine *Pasteurella multocida* isolates by MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA). *Sci Rep 8*, 13599.

Vanden Bush, T.J., Rosenbusch, R.F. 2002. *Mycoplasma bovis* induces apoptosis of bovine lymphocytes. *FEMS Immunol Med Microbiol 32*, 97-103.

Varga, C., Rajić, A., McFall, M.E., Reid-Smith, R.J., Deckert, A.E., Checkley, S.L., McEwen, S.A. 2009. Associations between reported on-farm antimicrobial use practices and observed antimicrobial resistance in generic fecal *Escherichia coli* isolated from Alberta finishing swine farms. Prev. *Vet Med 88*, 186-192.

Vereecke, N., Bokma, J., Haesebrouck, F., Nauwynck, H., Boyen, F., Pardon B., Sebastiaan, T. 2020. High quality genome assemblies of *Mycoplasma bovis* using a taxon-specific Bonito basecaller for MinION and Flongle long-read nanopore sequencing. *BMC Bioinformatics 21*, 517

Villarino, N., Brown, S.A., Martín-Jiménez, T. 2014. Understanding the pharmacokinetics of tulathromycin: A pulmonary perspective. *J Vet Pharmacol Ther 37*, 211-221.

Vyletělová, M. 2010. The survival of *Mycoplasma bovis* at different temperatures. *Czech J Food Sci 28,* 74-78.

Wawegama, N.K., Markham, P.F., Kanci, A., Schibrowski, M., Oswin, S., Barnes, T.S., Firestone, S.M., Mahony, T.J., Browning, G.F. 2016. Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for detection of *Mycoplasma bovis* infection in feedlot cattle. *J Clin Microbiol 54*, 1269-1275.

Welker, M., Moore, E.R.B. 2011. Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. *Syst Appl Microbiol 34*, 2-11.

White, D.G., Hudson, C., Maurer, J.J., Ayers, S., Zhao, S., Lee, M.D., Bolton, L., Foley, T., Sherwood, J. 2000. Characterization of chloramphenicol and florfenicol resistance in *Escherichia coli* associated with bovine diarrhea. *J Clin Microbiol 38*, 4593-4598.

Wiggins, M.C., Woolums, A.R., Hurley, D.J., Sanchez, S., Ensley, D.T., Donovan, D. 2011. The effect of various *Mycoplasma bovis* isolates on bovine leukocyte responses. *Comp Immunol Microbiol Infect Dis 34*, 49-54.

Wilson, D.J., Skirpstunas, R.T., Trujillo, J.D., Cavender, K.B., Bagley, C. V., Harding, R.L. 2007. Unusual history and initial clinical signs of *Mycoplasma bovis* mastitis and arthritis in first-lactation cows in a closed commercial dairy herd. *J Am Vet Med Assoc 230*, 1519-1523.

Wisselink, H.J., Smid, B., Plater, J., Ridley, A., Andersson, A.M., Aspán, A., Pohjanvirta, T., Vähänikkilä, N., Larsen, H., Høgberg, J., Colin, A., Tardy, F. 2019. A European interlaboratory trial to evaluate the performance of different PCR methods for *Mycoplasma bovis* diagnosis. *BMC Vet Res 15*, 86.

Wiuff, C., Lykkesfeldt, J., Aarestrup, F.M., Svendsen, O. 2002. Distribution of enrofloxacin in intestinal tissue and contents of healthy pigs after oral and intramuscular administrations. *J Vet Pharmacol Ther 25*, 335-342.

Wojciechowski, M., Czapinska, H., Bochtler, M. 2013. CpG underrepresentation and the bacterial CpG-specific DNA methyltransferase M. MpeI. *Proc Natl Acad Sci USA 110*, 105-110.

Wondji, C.S., Hemingway, J., Ranson, H. 2007. Identification and analysis of Single Nucleotide Polymorphisms (SNPs) in the mosquito *Anopheles funestus*, malaria vector. *BMC Genomics 8*, 5.

World Health Organization (WHO). 1985. The Rational Use of Drugs. Report of the Conference of Experts. Geneva.

World Health Organization (WHO). 2017. WHO Guidelines on use of medically important antimicrobials in food-producing animals. https://apps.who.int/iris/bitstream/handle/10665/258970/9789241550130-eng.pdf;jsessionid=10686E608DFDE39EE4630F7CD5A739C2?sequence=1

World Health Organization (WHO). 2020. SARS-CoV-2 Variants. https://www.who.int/csr/don/31-december-2020-sars-cov2-variants/en/ (last accessed March 17th 2021).

Yair, Y., Borovok, I., Mikula, I., Falk, R., Fox, L.K., Gophna, U., Lysnyansky, I. 2020. Genomics-based epidemiology of bovine *Mycoplasma bovis* strains in Israel. *BMC Genomics 21*, 70.

Yang, H.C., Chen, C.H., Wang, J.H., Liao, H.C., Yang, C.T., Chen, C.W., Lin, Y.C., Kao, C.H., Lu, M.Y.J., Liao, J.C. 2020. Analysis of genomic distributions of SARS-CoV-2 reveals a dominant strain type with strong allelic associations. *Proc Natl Acad Sci USA 117,* 30679-30686.

Zhang, N., Ye, X., Wu, Y., Huang, Z., Gu, X., Cai, Q., Shen, X., Jiang, H., Ding, H. 2017. Determination of the mutant selection window and evaluation of the killing of *Mycoplasma gallisepticum* by danofloxacin, doxycycline, tilmicosin, tylvalosin and valnemulin. *PLoS One 12*, e0169134.

Zhang, R., Han, X., Chen, Y., Mustafa, R., Qi, J., Chen, X., Hu, C., Chen, H., Guo, A. 2014. Attenuated *Mycoplasma bovis* strains provide protection against virulent infection in calves. *Vaccine 32*, 3107-3114.

Zhao, G., Zhu, X., Zhang, H., Chen, Y., Schieck, E., Hu, C., Chen, H., Guo, A. 2021. Novel secreted protein of *Mycoplasma bovis* MbovP280 induces macrophage apoptosis through CRYAB. *Front Immunol 12*, 619362.

Zimmermann, P. Ziesenitz, V.C., Curtis, N., Ritz, N. 2018. The immunomodulatory effects of macrolides – a systematic review of the underlying mechanisms. *Front Immunol 9*, 302.

CHAPTER 2

SCIENTIFIC AIMS

Mycoplasma bovis is the leading primary cause of pneumonia, arthritis and otitis in calves, but also causes arthritis and mastitis in adult cattle. *M. bovis* is highly contagious and affects multiple cattle production systems. Despite a growing body of epidemiological studies, little is known on molecular epidemiology and persistence within specific sectors. *M. bovis* associated diseases usually respond poorly to antimicrobial therapy, resulting in production losses, hampered animal welfare and excessive antimicrobial use. Next to inherent immune-evasive characteristics of the bacteria, the main reasons for treatment failure are late therapy initiation due to late detection and inappropriate antimicrobial treatment. *M. bovis* is often either naturally resistant (beta-lactam antibiotics and sulphonamides) or has acquired resistance against first choice antibiotics. For a better control and treatment success both rapid identification of *M. bovis* and antimicrobial susceptibility testing are key. Available diagnostic methods, like culturing techniques or PCR have several drawbacks, such as costs, diagnostic accuracy, interpretative issues and in particular a long sample-to-result turnaround time. To date, no routine antimicrobial susceptibility testing is performed and a reference framework for resistance determination is lacking.

Therefore, the general aim of this thesis was to develop new Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) and nanopore sequencing based diagnostic methods for rapid identification, strain typing, and antimicrobial susceptibility testing of *M. bovis*, and to apply those methods on Belgian field isolates and samples, gaining better insight into the epidemiology of *M. bovis*.

The specific objectives were:

- To explore and optimize *M. bovis* identification with MALDI-TOF MS from solid (Chapter 3.1) and liquid media (Chapter 3.2)
- To develop new methods for the rapid identification of *M. bovis* from broncho-alveolar lavage fluid with MALDI-TOF MS (Chapter 3.3) and nanopore sequencing (Chapter 3.4)
- To explore rapid nanopore sequencing as a new method for *M. bovis* strain typing and to map the spread of *M. bovis* strain types over Belgium and in different sectors (Chapter 4)
- To determine the antimicrobial susceptibility of recent Belgian *M. bovis* isolates from different cattle sectors (Chapter 5.1)
- To develop a rapid molecular method for antimicrobial susceptibility testing based on nanopore sequencing (Chapter 5.2)

CHAPTER 3

INNOVATIVE METHODS FOR THE IDENTIFICATION OF MYCOPLASMA BOVIS

NON-SPECIFIC, AGAR MEDIUM-RELATED PEAKS CAN RESULT IN FALSE POSITIVE *MYCOPLASMA ALKALESCENS* AND *MYCOPLASMA ARGININI* IDENTIFICATION BY MALDI-TOF MS

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ABSTRACT

MALDI-TOF MS is a fast and accurate tool to identify *Mycoplasma* species in liquid media. However, when trying to identify presumptive *Mycoplasma bovis* (*M. bovis*) colonies from solid medium (the "direct transfer method") a surprisingly high occurrence of *M. arginini* and *M. alkalescens* identification was observed. It was hypothesized that agar medium components are associated with false positive identification with *Mycoplasma* spp., as *M. bovis* colonies are very small and grow into the agar. The objective of this study was to determine whether complete modified pleuropneumonia-like organism (PPLO) agar (supplemented with horse serum, sodium pyruvate, technical yeast extract, ampicillin sodium salt and colistin) and the separate components, result in false identification as *Mycoplasma* spp. by MALDI-TOF MS. A total of 100 samples were examined, of which thirty-three percent of the modified PPLO agar spots were identified as *M. alkalescens* (16%) and *M. arginini* (17%)), albeit with relatively low score values (< 1.85). No false identification of *M. bovis* was obtained. Several medium components (unsupplemented PPLO agar, horse serum and colistin) resulted in spectra with peaks showing close matches with peaks present in the *M. alkalescens* and *M. arginini* database spectra. This study shows that the direct transfer method should be interpreted with caution, and one should strive to pick as little as possible agar when sampling *Mycoplasma*-like colonies from solid medium containing PPLO agar, horse serum and/or colistin.

Keywords: Colistin, Direct transfer method, Horse serum, PPLO agar

SHORT COMMUNICATION

Cattle are subject to diseases associated with multiple *Mycoplasma* species of which *Mycoplasma bovis* (*M. bovis*) is widely accepted as a primary pathogen (Maunsell and Donovan, 2009; Maunsell et al., 2011; Haapala et al., 2019; Oliveira et al., 2019). *M. bovis* is causing pneumonia, arthritis and otitis in calves (Maunsell and Donovan 2009), but also arthritis and mastitis in adult cattle (Maunsell et al., 2011). For other *Mycoplasma* spp. literature is more contradictory. *M. alkalescens* has been associated with arthritis in calves (Bennett et al., 1978; Whithear et al,. 1983) and mastitis in cattle (Jasper, 1982) in the past, whereas *M. arginini* has been described as an opportunistic pathogen (Shahriar et al., 2002; Thomas et al., 2002). *M. canadense* causes mastitis, and has been isolated from the lung (Jasper, 1977; Ball and Mackie, 1986). The pathogenicity of *M. bovirhinis* is questionable, as it is isolated from healthy and pneumonic lungs (Thomas et al., 2002). Also *M. dispar* was long thought to be pathogenic, but seems currently abundant in healthy animals (Timset et al., 2018). The impact of former *Mycoplasma* species in respiratory diseases is unknown (Thomas et al., 2002; Ayling et al., 2004), and may represent a part of the natural microbiome of the bovine respiratory tract (Klima et al., 2019; McMullen et al., 2019).

Rapid diagnosis and differentiation between *Mycoplasma* spp. is important, as *M. bovis* spreads easily, can be resistant against a lot of antimicrobials (both intrinsically and acquired) and is difficult to eradicate (Gautier-Bouchardon, 2018; Maunsell and Chase, 2019). Isolation of *M. bovis* from clinical samples requires nutrient rich media, often based on pleuropneumonia-like organisms (PPLO) agar, supplemented with animal serum, yeast extract and various antibiotics to prevent *M. bovis* of being overgrown by other bacteria. As for many other bacterial species, MALDI-TOF MS is a fast and accurate tool to identify *Mycoplasma* species, from a liquid medium (Pereyre et al., 2013; Randall et al., 2015; Spergser et al., 2019). However, most commonly, bacteria are cultured on solid medium and identified by MALDI-TOF MS by transferring colonies from the agar to the MALDI-TOF MS target plate ("direct transfer method") (Bizzini and Greub, 2010). In contrast to other bacteria, this direct transfer method appears to be unreliable for *M. bovis* identification (Pereyre et al., 2013). Culturing in broth and subsequent protein extraction are still necessary to achieve consistently high identification scores in *M. bovis* (Pereyre et al., 2013; Bokma et al., 2019). During attempts to identify presumptive *Mycoplasma*-like colonies obtained from broncho-alveolar lavages and pure *M. bovis* cultures with the direct transfer method, we observed a surprisingly high occurrence of *M. arginini* and *M. alkalescens* identifications

(data not shown). We noticed this especially in cases with very few and/or small colonies, suggesting that medium components might play a role. Therefore, the objective of this study was to explore whether agar medium components are associated with false positive *Mycoplasma* spp. identification using MALDI-TOF MS.

Five modified PPLO agar plates were produced in-house and contained PPLO agar (Difco™) supplemented with 25% inactivated horse serum (Gibco™), 0.5% sodium pyruvate (ReagentPlus, Sigma-Aldrich®), 0.7% technical yeast extract (Bacto™), 520 µg/mL ampicillin sodium salt (Sigma-Aldrich®) and 670 I.E./mL colistin (Colistine sulfate, VMD). The non-inoculated modified PPLO agar plates were incubated for 4 days (37ºC, 5% CO_2) and subsequently spotted 20 times per plate on a polished steel BC target plate with a toothpick using the direct transfer method. Additionally, we spotted the single components (PPLO agar, horse serum, yeast extract, pyruvate, ampicillin, and colistin) in duplicate to obtain reference spectra for the respective components. For colistin, both the injectable colistin sulfate (VMD) and a colistin sulfate standard (Sigma-Aldrich®) dissolved in distilled water to a concentration of 1 MIO I.E./ml, were included. Spotted samples were covered with one µl matrix (α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile - 47.5% water - 2.5% trifluoroacetic acid; Bruker Daltonics, Bremen) and processed with an Autoflex III (Bruker Daltonics, Bremen). Successful *Mycoplasma* species identification at species level was considered for logarithmic score values ≥ 1.7, as suggested earlier (Pereyre et al., 2013; Randall et al., 2015; Spergser et al., 2019). The standard Bruker library (server version 4.1.80 PYTH) was extended with four main spectrum profiles of recent *M. bovis* strains as described in Bokma et al. (2019). Peaks and spectra were analyzed with Flex-analysis 3.4 software after smoothing and baselining with the MBT_standard method and MALDI Biotyper Compass Explorer 4.1 (Bruker Daltonics, Bremen). Bruker Bacterial Test Standard (Bruker Daltonics, Bremen) was included in every run as quality control.

Thirty-three percent of the 100 spots with pure modified PPLO agar were as best match identified as *M. alkalescens* (16%) and *M. arginini* (17%), albeit with relatively low score values (1.70 – 1.82) (Supplement 1). In 7 out of 100 spots, both *M. alkalescens* and *M. arginini* were identified. We also observed a discrepancy between plates, as 7 out of 20 spots from plate 1 were false positive, wheras 7/20, 3/20, 5/20 and 12/20 were false positive from plate 2-5, respectively (Supplement 1). This variation might have been caused by slightly different concentrations of the agar components after solidifying the agar or the quantity of agar that was spotted on the target plate. Nonetheless, the results show that false positive identification of *M. alkalescens* and *M. arginini* was possible from every plate. No false

positive identification of *M. bovis* was obtained. The latter is in line with a previous study where false identification of *M. arginini* was detected with the direct transfer method from CHROMagar™ Orientation agar. However, in the previous study, *M. alkalescens* was never identified with relevant identification scores ≥ 1.7 (Lagacé-Wiens et al., 2019).

In order to gain information on which medium components might evoke the false positive results, the spectra of specific medium components were compared with peaks showing similar m/z [Da] values in the *M. alkalescens* 22B10 VLW and *M. arginini* 7SR10 VLW main spectrum profiles (MSP) in Bruker database records as these strains showed the highest matching identification scores with modified PPLO. As described in the metadata of the library, both MSPs of these *Mycoplasma* spp. were based on protein extraction after incubation for 3-5 days in Eaton's broth. Additionally, peaks of the entire modified PPLO agar were compared with *M. alkalescens* 22B10 VLW (Figure 1A) and *M. arginini* 7SR10 VLW (Figure 1B) MSPs.

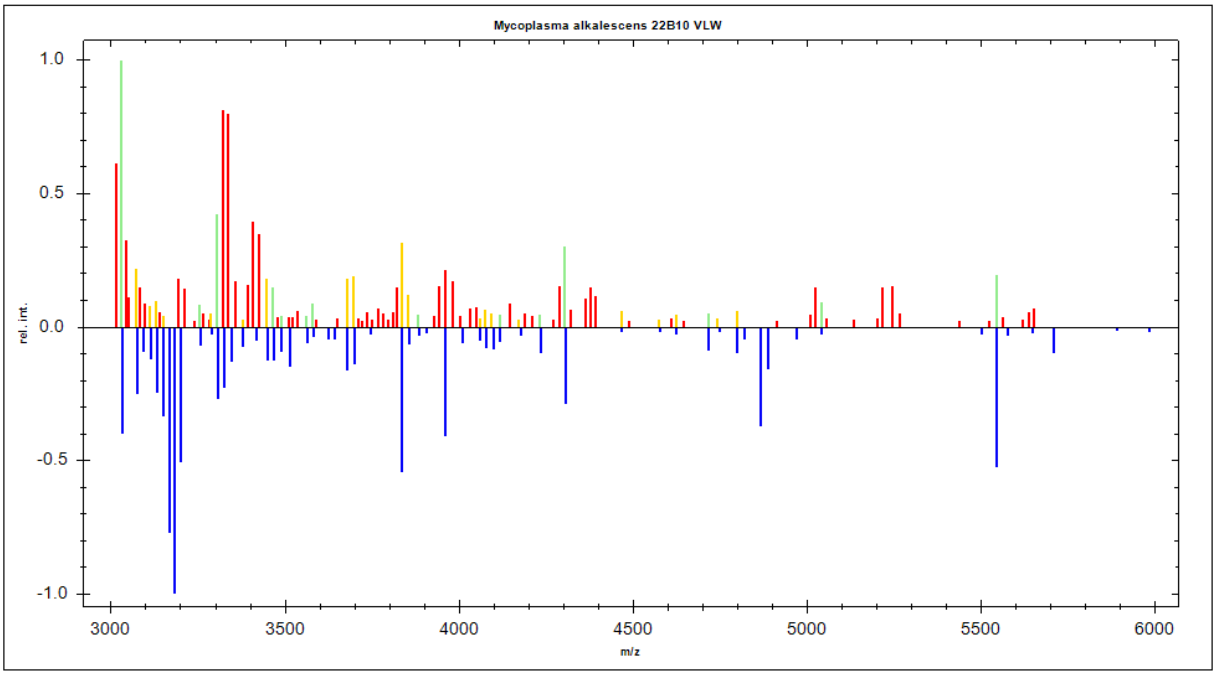

Figure 1A. Spectra comparison of modified PPLO agar (containing horse serum, yeast extract, pyruvate, ampicillin and colistin) (bars above the x-axis) with *Mycoplasma alkalescens* 22B10 VLW (blue bars) in MALDI Biotyper Compass Explorer 4.1 (Bruker Daltonics, Bremen). Peaks of matched spectra are depicted as green (peak match within experimental error range), orange (close peak match, but not within experimental error range) or red (no matching peaks).

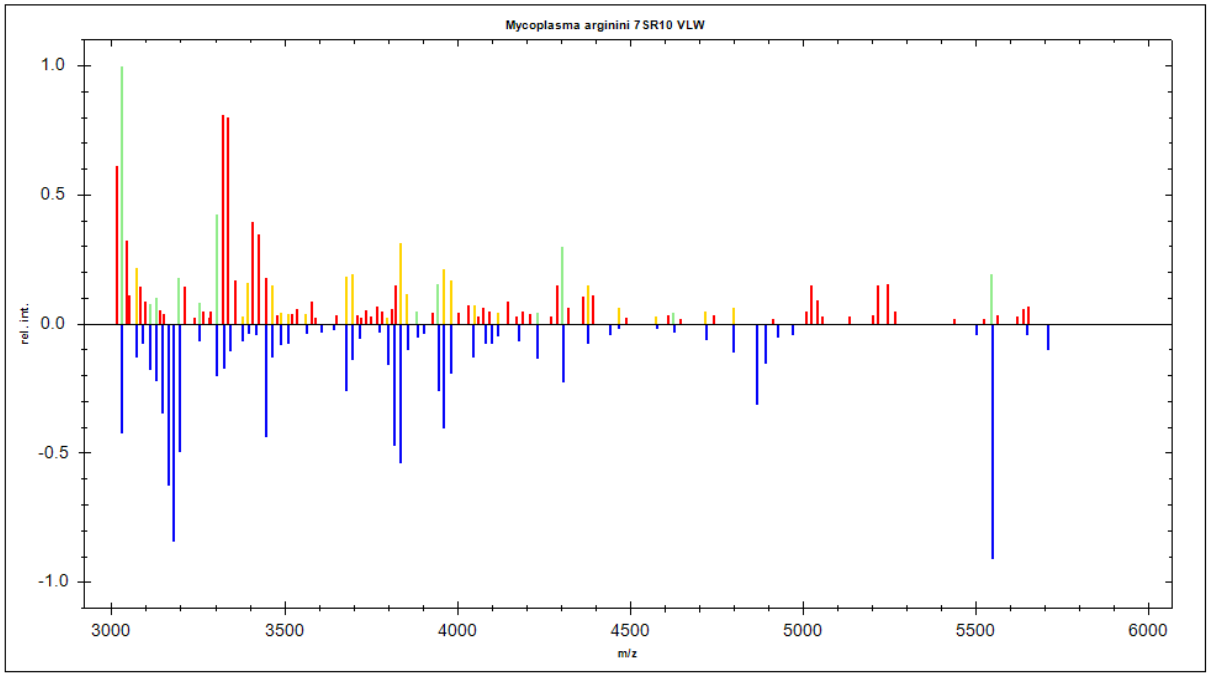

Figure 1B. Spectra comparison of modified PPLO agar (containing horse serum, yeast extract, pyruvate, ampicillin and colistin)(bars above the x-axis) with *Mycoplasma arginini* 7SR10 VLW (blue bars) in MALDI Biotyper Compass Explorer 4.1 (Bruker Daltonics, Bremen). Peaks of matched spectra are depicted as green (peak match within experimental error range), orange (close peak match, but not within experimental error range) or red (no matching peaks).

When analyzing the separate components, only PPLO agar, horse serum and colistin (both injectable and pure form) resulted in meaningful MALDI-TOF spectra. The components together showed 39 (close) matching peaks with the investigated *Mycoplasma* spp. (7 for PPLO agar, 3 for horse serum and 32 for colistin) based on m/z [Da] values between 3000 – 15.000 (as used for identification), as shown in Table 1. Using Compass Explorer software, peaks of matched spectra are depicted as green (peak match within experimental error range), orange (close peak match, but not within experimental error range) or red (no matching peaks), as defined by the manufacturer. When comparing modified PPLO agar with *M. alkalescens* 22B10 VLW, 14 peaks matched within experimental error range (green) and 20 were close peak matches (orange), for *M. arginini* 7SR10 this was 12 and 21, respectively. These overlapping peaks might vary sometimes due to small variations in spectra caused by the sample quality or when intensity of a peak is on the borderline of detection.

No false positive *M. bovis* identification was observed with the current protocol. Nevertheless, false positive *Mycoplasma* spp. results could lead to unnecessary antimicrobial use in animals, risking increased antimicrobial resistance selection (Chantziaras et al., 2014; Tang et al., 2017).

This study showed that to a large extend, spectrum peaks originating from PPLO agar, horse serum and colistin can result in false positive identification of *M. alkalescens* and *M. arginini*. This is in contrast to an earlier study where it was hypothesized, though not investigated, that false positive *Mycoplasma* identifications were a result of yeast extract or peptone related peaks (Lagacé-Wiens et al., 2019). Nevertheless, not all PPLO agar, colistin and horse serum related peaks could explain the overlapping peaks between modified PPLO agar and the *Mycoplasma* spp. Therefore, it could be possible that yeast extract or peptones show indeed peaks, but intensity might be too low for MALDI-TOF MS to generate a distinguishable spectrum when investigating the separate agents.

One of the reasons that *M. alkalescens* and *M. arginini* were identified, while other *Mycoplasma* spp. cultivated in the same medium were not, could be the use of an imperfect protocol of generating these specific MSPs. For example, the use of *Mycoplasma* cultures with too low bacterial biomass, due to either concentration limits or phase requirements in broth culture or inefficient washing steps, may have contributed to low quality protein extractions. In addition, it was recently described that ethanol protein precipitation for *Mycoplasma* spp. may lead to unstable quality of spectra (Spergser et al., 2019). It is very probable that ethanol was used when obtaining older MSPs, even though it is not clear whether this could have contributed to differences between species. Finally, mere chance may have resulted in the fact that some *M. alkalescens* and *M. arginini* peptides/proteins share molecular masses with certain medium components, while this might not be the case for other *Mycoplasma* spp. Replacing the current library MSPs by new ones obtained with another protocol (for example including efficient washing steps and without ethanol precipitation) might be effective. Alas, the present software does not provide the liberty to replace MSPs.
As alternative technique to reduce the amount of agar attached to the *Mycoplasma* colonies, the authors explored the use of a plastic loop instead of a toothpick. Unfortunately, it was almost impossible to gather enough material for reliable peak recognition with MALDI-TOF MS. Currently, the best option to identify *Mycoplasma* spp. with MALDI-TOF MS seems to be from broth cultures (Spergser et al., 2019., Bokma et al., 2019). Misidentifications probably do not occur starting from broth cultures, as they are usually washed and therefore medium-related proteins are removed prior to protein extraction. This results in clearer bacterial peaks and less background noise. Also *Mycoplasma* spp. concentrations are likely higher in liquid medium compared with what can be obtained from picking one colony from

an agar plate. Therefore, the balance between bacterial matter and medium components is expected to be better starting from broth cultures.

Table 1. Overview of spectra (m/z)[Da] showing peaks of horse serum and colistin (injectable and standard form) similar to *M. alkalescens* 22B10 VLW and *M. arginini* 7SR10 VLW (within a deviation range of 10 Da)

PPLO agar	*M alkalescens* 22B10 VLW	*M. arginini* 7SR10 VLW	Colistin sulfate	*M alkalescens* 22B10 VLW	*M. arginini* 7SR10 VLW	Horse serum	*M alkalescens* 22B10 VLW	*M. arginini* 7SR10 VLW
3024	3031	3028	3024		3028			
			3036	3031				
			3072	3073	3071			
			3087	3091	3090			
			3106	3113	3111			
			3128	3132	3129			
			3148	3151	3148			
			3170	3168				
			3187	3183				
			3195	3200	3195			
			3290	3288				
3319	3326	3324						
3334		3334						
			3412	3416	3416			
			3451	3448	3445			
			3460	3465	3465			
			3488	3489	3487			
			3507		3511			
			3679	3679	3678			
			3717		3717			
			3745	3745				
3979		3979	3980		3979			
			4010	4009				
			4043		4046			
			4065	4061				
			4085	4078	4079			
			4099	4097	4097			
			4114	4118	4115			
			4120	1118				
			4177	4176	4175			
			4178	4176				
			4232	4232	4232			
4300	4304	4305	4304	4304	4305			
			4470	4468	4467			
						4715	4717	4718
						5542	5544	5545
						5580	5574	
6804		6805						
6821	6815							

All peaks were extracted from the mass list generated by Flex-analysis 3.4 software after smoothing and baselining the initial spectra generated by MALDI-TOF MS (Bruker Daltonics, Bremen)

The present study shows that MALDI-TOF identification results using the direct transfer method from solid media for *Mycoplasma* spp., which allows identification of bacteria within several minutes and with minimal labor, should be interpreted cautiously in diagnostic laboratories. One should strive to pick enough bacterial material with as little as possible agar, especially when colonies are small (higher risk for including agar) and when PPLO agar, colistin and/or horse serum is added to the medium. This should not only be kept in mind for identification with MALDI-TOF MS, but also when generating new MSP's to include in the library.

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SUPPLEMENTARY DATA

Supplement 1. MALDI-TOF MS identification scores of *Mycoplasma* species per sample, can be found online at https://doi.org/10.1016/j.rvsc.2020.03.010.

REFERENCES

Ayling, R.D., Bashiruddin, S.E., Nicholas, R.A. 2004. *Mycoplasma* species and related organisms isolated from ruminants in Britain between 1990 and 2000. *Vet Rec 155*, 413-416.

Ball, H.J., Mackie, D.P. 1986. Experimental production of bovine and ovine mastitis with a *Mycoplasma canadense* isolate. *Vet Rec 118,* 72-72.

Bennett, R.H., Jasper, D.E. 1978. *Mycoplasma alkalescens*-induced arthritis in dairy calves. *J Amer Vet Med Assoc 172*, 484-488.

Bizzini, A., Greub, G. 2010. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. *Clin Microbiol Infect 16*, 1614-1619.

Bokma, J., Pardon, B., Van Driessche, L., Gille, L., Deprez, P., Haesebrouck, F., Boyen, F. 2019. Optimizing identification of *Mycoplasma bovis* by MALDI-TOF MS. *Res Vet Sci 125*, 185-188.

Chantziaras, I., Boyen, F., Callens, B., Dewulf, J. 2014. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. J Glob Antimicrob Resist 69, 827-834.

Gautier-Bouchardon, A.V. 2018. Antimicrobial Resistance in *Mycoplasma* spp. *Microbiol Spectr 6*.

Haapala, V., Herva, T., Härtel, H., Pitkänen, E., Mattila, J., Rautjoki, P., Pelkonen, S., Soveri, T., Simojoki, H. 2019. Comparison of Finnish meat inspection records and average daily gain for cattle herds differing in *Mycoplasma bovis* test-status. *Vet J 249*, 41-46.

Jasper, D.E. 1977. Mycoplasma and mycoplasma mastitis. *J Am Vet Med Assoc 170*, 1167-1172.

Jasper, D.E. 1982. The role of Mycoplasma in bovine mastitis. *J Amer Vet Med Assoc 181*, 158-162.

Klima, C.L., Holman, D.B., Ralston, B.J., Stanford, K., Zaheer, R., Alexander, T.W., McAllister, T.A. 2019. Lower respiratory tract microbiome and resistome of bovine respiratory disease mortalities. *Microb Ecol 78*, 446-456.

Lagacé-Wiens, P.R.S., Abbott, A.A., Karlowsky, J.A. 2019. CHROMagar™ orientation urine culture medium produces matrix-assisted laser desorption ionization-time-of-flight mass spectrometry spectra misidentified as *Mycoplasma arginini* and *Mycoplasma alkalescens*. *Diagn Microbiol Infect Dis 94*, 113-115.

Maunsell, F.P., Chase, C. 2019. *Mycoplasma bovis* interactions with the immune system and failure to generate an effective immune response. *Vet Clin Food Anim 35*, 471-483.

Maunsell, F.P., Donovan, G.A. 2009. *Mycoplasma bovis* infections in young calves. *Vet Clin North Am Food Anim Pract 25*, 139-177.

Maunsell, F.P., Woolums, A.R., Francoz D., Rosenbusch R.F., Step, D.L., Wilson, D.J., Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle. *J Vet Intern Med 25*, 772-783.

McMullen, C., Orsel, K., Alexander, T.W., van der Meer, F., Plastow, G., Timsit, E. 2019. Comparison of the nasopharygneal bacterial microbiota of beef calves raised without the use of antimicrobials between healthy calves and those diagnosed with bovine respiratory disease. *Vet Mic 231*, 56-62.

Oliveira, T.E.S., Pelaquim, I.F., Flores E.F., Massi, R.P., Valdiviezo, M.J.J., Pretto-Giordano, L.G., Alfieri, A.A., Saut, J.P.E., Headley, S.A. 2019. *Mycoplasma bovis* and viral agents associated with the development of bovine respiratory disease in adult dairy cows. *Transbound Emerg Dis*. doi: 10.1111/tbed.13223

Pereyre, S., Tardy, F., Renaudin, H., Cauvin, E., Del Pra Netto Machado, L., Tricot, A., Benoit, F., Treilles, M., Bebear, C. 2013. Identification and subtyping of clinically relevant human and ruminant mycoplasmas by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol 51*, 3314-3323.

Randall, L.P., Lemma, F., Koylass, M., Rogers, J., Ayling, R.D., Worth, D., Klita, M., Steventon, A., Line, K., Wragg, P., Muchowski, J., Kostrzewa, M., Whatmore, A.M. 2015. Evaluation of MALDI-TOF as a method for the identification of bacteria in the veterinary diagnostic laboratory. *Res Vet Sci 101*, 42-49.

Shahriar, F.M., Clark, E.G., Janzen, E., West, K., Wobeser, G. 2002. Coinfection with bovine viral diarrhea virus and *Mycoplasma bovis* in feedlot cattle with chronic pneumonia. *Can Vet J 43,* 863-868.

Spergser, J., Hess, C., Loncaric, I., Ramírez, A.S. 2019. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry is a superior diagnostic tool for the identification and differentiation of Mycoplasmas isolated from animals. *J Clin Microbiol 57*, e00316-19.

Tang, K.L., Caffrey, N.P., Nóbrega, D.B., Cork, S.C., Ronksley, P.E., Barkema, H.W., Polachek, A.J., Ganshorn, H., Sharma, N., Kellner, J.D., Ghali, W.A. 2017. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. *Lancet Planet Health 1*, e316-327.

Timsit, E., Workentine, M., van der Meer, F., Alexander, T. 2018. Distinct bacterial metacommunities inhabit the upper and lower respiratory tracts of healthy feedlot cattle and those diagnosed with bronchopneumonia. *Vet Mic 221*, 105-113.

Thomas, A., Ball, H., Dizier, I., Trolin, A., Bell, C., Mainil, J., Linden, A. 2002. Isolation of *Mycoplasma* species from the lower respiratory tract of healthy cattle and cattle with respiratory disease in Belgium. *Vet Rec 151*, 472-476.

Whithear, K.G. 1983. Isolation of *Mycoplasma alkalescens* from cases of polyarthritis in embryo-transplant calves. *Aust Vet J 60*, 191-192.

OPTIMIZING IDENTIFICATION OF *MYCOPLASMA BOVIS* BY MALDI-TOF MS

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ABSTRACT

Fast and accurate identification of *Mycoplasma bovis* in cattle samples is of great importance for rational treatment and control of pneumonia, arthritis and mastitis. However, which growth conditions will allow for the fastest identification of *M. bovis* with MALDI-TOF MS remains unclear. Therefore, growth conditions and incubation time were investigated to optimize identification of *M. bovis* with MALDI-TOF MS and an in-house library was constructed. Nine different *M. bovis* strains were inoculated in triplicate in three liquid media (B1-3). Basic broth (B1) consisted of pleuropneumonia-like organism broth, enriched with 25% horse serum and 0.7% yeast extract. B2 and B3 were additionally supplemented with 0.5% pyruvate or 520 µg/mL ampicillin, respectively. Protein extraction was performed after 24, 48, 72, 96 and 120 hours of incubation (37°C, 5% CO_2) and processed with Autoflex III smartbeam. Identification scores ≥1.7 were interpreted as reliable. The present study showed reliable identification of *M. bovis* with MALDI-TOF MS as early as 24h after inoculation, and in broth supplemented with pyruvate, up to 120h after inoculation. Serial dilutions showed improved survival of *M. bovis* in broth with pyruvate. The addition of ampicillin to prevent contamination, did not impair identification of *M. bovis* and state-of-the-art in-house libraries contributed to higher identification scores for *M. bovis* with MALDI-TOF MS.

Keywords: ampicillin, incubation time, library, protein extraction, pyruvate

SHORT COMMUNICATION

Mycoplasma bovis (*M. bovis*) is an important pathogen causing primarily pneumonia, otitis and arthritis in calves, and pneumonia and mastitis in adult cattle (Maeda et al., 2003; Maunsell and Donovan, 2009; Maunsell et al., 2011). Mixed infections of *M. bovis* with other less or apathogenic *Mycoplasma* or *Acholeplasma* species, such as *Mycoplasma bovirhinis, Mycoplasma arginini* and *Acholeplasma laidlawii* can be present in bovine clinical samples (Thomas et al., 2002; Ayling et al., 2004; Autio et al., 2007; Szacawa et al., 2018). While cultivation is inexpensive and allows further characterization of the isolates, such as susceptibility testing and strain typing, definite identification requires other techniques, such as PCR methods (Calcutt et al., 2018; Parker et al., 2018). Nevertheless, fast identification of *M. bovis* is highly important in order to rationalize antimicrobial use, since *M. bovis* is inherently resistant against various antimicrobials, such as penicillins, cephalosporins and (potentiated) sulphonamides, often used to treat bovine respiratory disease (Gautier-Bouchardon, 2018; Nicholas and Ayling, 2003). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a fast and reliable technique for identifying bacteria until species level (Bizzini and Greub, 2010; Kuhnert et al., 2012; Puchalski et al., 2016). Even though spectra peaks are mainly originating from ribosomal proteins (Sauer and Kliem, 2010), quality and reproducibility of obtained spectra can be influenced by different circumstances, e.g. growth medium and incubation time (Welker and Moore, 2011; Anderson et al., 2012; Martiny et al., 2013). Although the identification of *M. bovis* with MALDI-TOF MS has been described earlier (Pereyre et al., 2013; Becker et al., 2015; Randall et al., 2015), it remains unclear what the best growth conditions are allowing for the fastest and most reliable identification of *M. bovis* for clinical applications. Therefore, the objective of this study was to investigate whether growth media, incubation time and used library influence identification of *M. bovis* with MALDI-TOF MS.

One to three colonies of nine different clinical bovine *M. bovis* strains (K6, Mb51, Mb99, Mb219, VK1, VK13, VK27, Mb263, Mb274; Table 1) were inoculated in triplicate in 25 mL of three liquid media (B1-3). As *M. bovis* is a fastidious grower, the basic broth (B1) consisted of Difco™ pleuropneumonia-like organism broth, enriched with 25% inactivated horse serum (Gibco™) and 0.7% technical yeast extract (Bacto™). B2 and B3 were additionally supplemented with 0.5% sodium pyruvate (ReagentPlus, Sigma-Aldrich®) or 520 µg/mL ampicillin sodium salt (Sigma-Aldrich®), respectively. Hypothesis was that supplementing pyruvate might improve growth rate and protein expression, since adding

pyruvate resulted in increased growth yield in non-fermenting *Mycoplasma* spp. before (Miles et al., 1988). Ampicillin was investigated as this antibiotic is often used to prevent contamination and bacterial overgrowth (Sachse et al., 1993).

Table 1. Descriptives of the twelve *Mycoplasma bovis* strains obtained in Belgium and used in this study.

Name	Sample	Herd	Region	Year	MSP
K1	Bronchoalveolar lavage	Veal	Antwerp	2014	Yes
K3	Bronchoalveolar lavage	Veal	Limburg	2014	Yes
K6	Bronchoalveolar lavage	Beef	East-Flanders	2014	Yes
K7	Bronchoalveolar lavage	Beef	East-Flanders	2014	Yes
Mb51	Bronchoalveolar lavage	Beef	Antwerp	2016	No
Mb99	Middle ear	Dairy	Limburg	2017	No
Mb219	Bronchoalveolar lavage	Dairy	East-Flanders	2017	No
VK1	Deep nasal swab	Veal	Antwerp	2017	No
VK13	Deep nasal swab	Veal	Antwerp	2017	No
VK27	Deep nasal swab	Veal	East-Flanders	2017	No
Mb263	Joint	Dairy/Beef	West-Flanders	2018	No
Mb274	Milk	Dairy	Henegouwen	2018	No

MSP = main spectrum profiles added to the in-house library

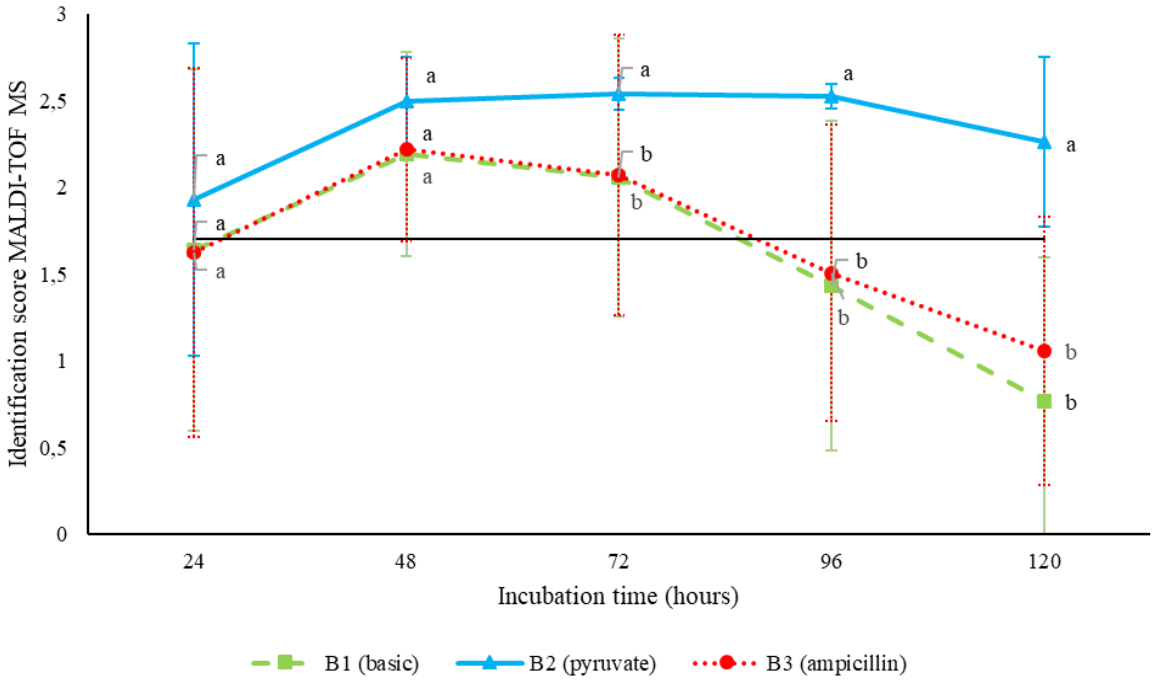
One mL aliquots were derived from the cultures, and protein extraction was performed as previously described at 24, 48, 72, 96 and 120 hours of incubation (37°C, 5% CO_2) (Pereyre et al., 2013). One µL of the protein extract was spotted in triplicate, air dried and covered with 1 µL alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Bremen, Germany) on a MSP 384 target polished steel BC plate. All samples were processed with an Autoflex III smartbeam MALDI-TOF MS, using FlexControl and MBT Compass software (Bruker Daltonics, Bremen, Germany). External calibration and validation were performed by adding Bacterial Test Standard, as described by the manufacturer (Bruker Daltonics). Spectra obtained during the experiments were compared to the standard Bruker library (SL; including *M. bovis* 86B96 VLW and *M. bovis* NCTC 10131T VLW) and the in-house library (IHL). The IHL consisted of the SL which was extended with four MSP's (main spectra profiles) from clinical bovine *M. bovis* isolates by Bruker's protocol to improve identification scores (Rettinger et al., 2012; Schulthess et al., 2016). Strains used to construct MSP's (K1, K3, K6, K7) were obtained from calves with pneumonia and identified using real-time PCR (Clothier et al., 2010) (Table 1). At the same time points aliquots were obtained for MALDI-TOF analysis, an aliquot was obtained for performing bacterial cell counts for five strains (VK13, Mb51, Mb99, Mb263, Mb274) in order to determine the concentration of *M. bovis* at the moment of protein extraction. Fifty µL of the dilution was inoculated on PPLO-agar plates

and counted manually after five to seven days of incubation (37°C, 5% CO_2) on plates showing 20-200 colonies per plate.

Identification score (ID-score) per spot were obtained and successful identification was considered at ID-scores ≥ 1.7, as proposed in previous studies (Pereyre et al., 2013; Randall et al., 2015; Schulthess et al., 2016). Spots were excluded as being contaminated, when a non-*M. bovis* ID was obtained within the first 10 matches with ID score values ≥ 1.7. All data were transferred to SAS 9.4 (SAS Institute Inc., Cary, N.C.). Data was analyzed by both a linear mixed regression model with the ID-score as continuous outcome variable and a logistic mixed regression models with ID-scores as a binary outcome variable (0 < 1.7; 1 ≥ 1.7). In both models, time was included as repeated effect to adjust for the repeated measurements within an isolate. Also, broth and isolate were in both models included as categorical predictor variables. Significance was set at $P < 0.05$.

Due to contamination, possibly caused by preserving contaminated samples during the experiment and a high number of technical repetitions, 5.2% (103/1980) of the readings (1 spot at 24h; 13 at 96h; 89 at 120h after incubation) were excluded from the statistical analysis. Therefore, cross contamination could be a potential drawback for long incubation. Statistical analysis showed significant differences in ID-score between broths ($P < 0.001$), which is in line with previous studies in other bacterial pathogens (Valentine et al., 2005; Anderson et al., 2012; Martiny et al., 2013). No significant strain effects were identified ($P = 0.11$). After 24 and 48h of incubation, the ID-score using the IHL was, although not statistically significant, superior in B2, in contrast to B1 (24h: $P = 0.15$; 48h: $P = 0,10$), and B3 ($P = 0.18$; $P = 0.27$). After 72h of incubation, ID-scores reduced drastically for B1 and B3, which has also been reported for *Campylobacter* (Martiny et al., 2013). In contrast, ID-scores for B2 remained similar until the end of the experiment (Figure 1). Also the percentage of correct identification was consistently higher in B2 after different incubation times (24h: 83%, 48h: 99%, 72h: 100%, 96h: 100% and 120h: 92%) compared to B1 (68%, 92%, 87%, 67% and 15%) and B3 (68%, 92%, 86%, 69% and 18%). The possible effects of pyruvate on *M. bovis* growth and protein expression has been described before (Miles et al., 1988; Masukagami et al., 2017), nevertheless results of the bacterial cell counts, showed that pyruvate did not improve *M. bovis* growth during the first 48h of incubation, but facilitated the survival of *M. bovis*. Cell counts remained more or less stable in B2, compared with B1 and B3 after 48-120 hours of incubation (Figure 1). In addition, even though not statistically significant, the MALDI-TOF score values did seem slightly higher and more robust using B2. The statistically significant stabilising effect of pyruvate at time points after 48h of incubation, might have contributed to

the seemingly more robust results at 24 and 48h of incubation. Lack of statistical significance at these earlier time points may be due inter-strain variation, because score values were already quite high for B1 and B3, or due to the fact that supplementing pyruvate actually does not have an added value at these earlier time points. Masukagami et al. (2017) showed that incorporation of pyruvate could not be detected in *M. bovis* in the very early logarithmic growth phase (after 4h of incubation). However, the earliest sampling point in this study is at 24h of incubation, and the most obvious effects are even seen after more than 48h of incubation.



	24h	SD	48h	SD	72h	SD	96h	SD	120h	SD
B1 (basic)	$2.0x10^8$	$2.8x10^8$	$1.7x10^8$	$7.5x10^7$	$1.6x10^8$	$1.2x10^8$	$3.9x10^7$	$5.3x10^7$	$1.1x10^5$	$1.5x10^5$
B2 (pyruvate)	$2.2x10^7$	$2.5x10^7$	$2.6x10^8$	$2.1x10^8$	$2.2x10^8$	$1.5x10^8$	$2.2x10^8$	$7.5x10^7$	$3.0x10^7$	$3.5x10^7$
B3 (ampicillin)	$1.4x10^8$	$1.0x10^8$	$1.3x10^8$	$8.2x10^7$	$1.7x10^8$	$9.6x10^7$	$3.7x10^7$	$2.3x10^7$	$1.3x10^5$	$1.2x10^5$

Figure 1. Evolution of MALDI-TOF MS identification scores of 9 *Mycoplasma bovis* strains (K6, Mb51, Mb99, Mb219, VK1, VK13, VK27, Mb263 and Mb274) after protein extraction at different incubation times, using the in-house library. Identification scores ≥ 1.7 (horizontal black line) were interpreted as reliable. Basic broth 1 (B1: green filled square), broth 2, supplemented with pyruvate (B2: blue filled triangle), broth 3, supplemented with ampicillin (B3: red filled circle). Vertical line presents standard deviation, based on 132 repetitions minus contaminated samples. Different superscripts$^{(a,b)}$ mean significantly different identification scores at one time point ($P < 0.001$). The table below presents *M. bovis* concentrations (CFU/ml) and standard deviation (SD) based on 5 *M. bovis* strains (Mb51, Mb99, Mb263, Mb274, VK13) in different broths at 24 to 120 hours of incubation.

Reliable identification of *M. bovis* with MALDI-TOF MS was possible for 83% of the samples as early as 24h after inoculation with one to three colonies obtained from an agar plate. This is as quick or even faster than other techniques as growth inhibition, immunofluorescence or dot immunobinding, used for identification after isolation (Devriese and Haesebrouck, 1991; Calcutt et al., 2018). PCR of the initial sample would be faster (Sachse et al., 1993), but to a higher cost, and lacks the opportunity for additional research on clinical isolates (Parker et al., 2018). Using a pyruvate supplemented broth, successful identification of *M. bovis* can be expected up to 120h after inoculation. This could be useful in routine diagnostics and research, when for example weekends and holiday's need to be overcome. Supplementation of ampicillin did not influence the ID-score, as expected, since *M. bovis* is inherently resistant against this antimicrobial drug (Gautier-Bouchardon, 2018). Therefore, supplementation of ampicillin could be useful in conditions where contamination is considered likely, without affecting MALDI-TOF MS identification efficiency.
The IHL showed higher ID-scores in broth 1, 2 and 3 after 48h (2.33; 2.51 and 2.33, respectively) and 72h (2.36; 2.54 and 2.37, respectively) of inoculation compared to the SL (48h: 1.75; 1.64; 1.77/72h: 1.77; 1.67; 1.77). The observed diversity between ID-scores for SL and IHL could be due to the genetic variation of *M. bovis* (McAuliffe et al., 2004; Amram et al., 2013; Becker et al., 2015).

In conclusion, this study showed that adding pyruvate to the medium ensured reliable identification with MALDI-TOF MS after 24 hours of incubation, persisting up to five days after inoculation. Additionally, adding ampicillin to the medium may be useful in avoiding contamination, as it does not undermine accurate identification with MALDI-TOF MS. Also expanding the MSP library could contribute to better identification of *M. bovis*, as the use of a state-of-the-art in-house library can contribute to higher score values. This information can be useful to improve MALDI-TOF MS assisted diagnosis of *M. bovis* in veterinary diagnostic laboratories.

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REFERENCES

Amram, E., Freed, M., Khateb, N., Mikula, I., Blum, S., Spergser, J., Sharir, B., Ozeri, R., Harrus, S., Lysnyansky, I. 2013. Multiple locus variable number tandem repeat analysis of *Mycoplasma bovis* isolated from local and imported cattle. *Vet J 197*, 286-290.

Anderson, N.W., Buchan, B.W., Riebe, K.M., Parsons, L.N., Gnacinski, S., Ledeboer, N.A. 2012. Effects of solid-medium type on routine identification of bacterial isolates by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol 50*, 1008-1013.

Autio, T., Pohianvirta, T., Holopainen, R., Rikula, U., Pentikäinen, J., Huovilainen, A., Rusanen, H., Soveri, T., Sihvonen, L., Pelkonen, S. 2007. Etiology of respiratory disease in non-vaccinated, non-medicated calves in rearing herds. *Vet Microbiol 119*, 256-265.

Ayling, R.D., Bashiruddin, S.E., Nicholas, R.A. 2004. *Mycoplasma* species and related organisms isolated from ruminants in Britain between 1990 and 2000. *Vet Rec 155*, 413-416.

Becker, C.A.M., Thibault, F.M., Arcangioli, M.A., Tardy, F. 2015. Loss of diversity within *Mycoplasma bovis* isolates collected in France from bovines with respiratory diseases over the last 35 years. *Infection Genetics and Evolution 33*, 118-126.

Bizzini, A., Greub, G. 2010. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. *Clin Microbiol Infect 16*, 1614-1619.

Calcutt, M.J., Lysnyansky, I., Sachse, K., Fox, L.K., Nicholas, R.A.J., Ayling, R.D. 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. *Transbound Emerg Dis 65* , 91-109.

Clothier, K.A., Jordan, D.M., Thompson, C.J., Kinyon, J.M., Frana, T.S., Strait, E.L. 2010. *Mycoplasma bovis* real-time polymerase chain reaction assay validation and diagnostic performance. *J Vet Diagn Invest 22*, 956-960.

Devriese, L.A., Haesebrouck, F. 1991. Antibiotic susceptibility testing of *Mycoplasma bovis* using Tween 80 hydrolysis as an indicator of growth. *Zentralbl Veterinarmed B 38*, 781-783.

Gautier-Bouchardon, A.V. 2018. Antimicrobial Resistance in *Mycoplasma* spp. *Microbiol Spectr* 6.

Kuhnert, P., Bisgaard, M., Korczak, B.M., Schwendener, S., Christensen, H., Frey, J. 2012. Identification of animal *Pasteurellaceae* by MALDI-TOF mass spectrometry. *J Microbiol Methods 89*, 1-7.

Maeda, T., Shibahara, T., Kimura, K., Wada, Y., Sato, K., Imada, Y., Ishikawa, Y., Kadota, K. 2003. *Mycoplasma bovis*-associated suppurative otitis media and pneumonia in bull calves. *J Comp Pathol 129*, 100-110.

Martiny, D., Visscher, A., Catry, B., Chatellier, S., Vandenberg, O. 2013. Optimization of *Campylobacter* growth conditions for further identification by matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). *J Microbiol Methods 94*, 221-223.

Masukagami, Y., De Souza, D.P., Dayalan, S., Bowen, C., O'Callaghan, S., Kouremenos, K., Nijagal, B., Tull, D., Tivendale, K.A., Markham, P.F., McConville, M.J., Browning, G.F., Sansom, F.M. 2017. Comparative metabolomics of *Mycoplasma bovis* and *Mycoplasma gallicsepticum* reveals fundamental differences in active metabolic pathways and suggests novel gene annotations. *mSystems 5*, e00055-17.

Maunsell, F.P., Donovan, G.A. 2009. *Mycoplasma bovis* infections in young calves. *Vet Clin North Am Food Anim Pract 25*, 139-177.

Maunsell, F.P., Woolums, A.R., Francoz D., Rosenbusch R.F., Step, D.L., Wilson, D.J., Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle. *J Vet Intern Med 25*, 772-783.

McAuliffe, L., Kokotovic, B., Ayling, R.D., Nicholas, R.A. 2004. Molecular epidemiological analysis of *Mycoplasma bovis* isolates from the United Kingdom shows two genetically distinct clusters. *J Clin Microbiol 42*, 4556-4565.

Miles, R.J., Wadher, B.J., Henderson, C.L., Mohan, K. 1988. Increased growth yields of *Mycoplasma* spp. in the presence of pyruvate. *Letters in Applied Microbiology* 7, 149-151.

Nicholas, R.A., Ayling, R.D. 2003. *Mycoplasma bovis*: disease, diagnosis, and control. *Res Vet Sci 74*, 105-112.

Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House, J.K. 2018. A review of mycoplasma diagnostics in cattle. *J Vet Intern Med 32*, 1241-1252.

Pereyre, S., Tardy, F., Renaudin, H., Cauvin, E., Del Pra Netto Machado, L., Tricot, A., Benoit, F., Treilles, M., Bebear, C. 2013. Identification and subtyping of clinically relevant human and ruminant mycoplasmas by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol 51*, 3314-3323.

Puchalski, A., Urban-Chmiel, R., Dec, M., Stegierska, D., Wernicki, A. 2016. The use of MALDI-TOF mass spectrometry for rapid identification of *Mannheimia haemolytica. J Vet Med Sci 78*, 1339-1342.

Randall, L.P., Lemma, F., Koylass, M., Rogers, J., Ayling, R.D., Worth, D., Klita, M., Steventon, A., Line, K., Wragg, P., Muchowski, J., Kostrzewa, M., Whatmore, A.M. 2015. Evaluation of MALDI-TOF as a method for the identification of bacteria in the veterinary diagnostic laboratory. *Res Vet Sci 101*, 42-49.

Rettinger, A., Krupka, I., Grunwald, K., Dyachenko, V., Fingerle, V., Konrad, R., Raschel, H., Busch, U., Sing, A., Straubinger, R.K., Huber, I. 2012. *Leptospira* spp. strain identification by MALDI TOF MS is an equivalent tool to 16S rRNA gene sequencing and multi locus sequence typing (MLST). *BMC Microbiol 12*, 185.

Sachse, K., Pfutzner, H., Hotzel, H., Demuth, B., Heller, M., Berthold, E. 1993. Comparison of various diagnostic methods for the detection of *Mycoplasma bovis*. *Rev Sci Tech 12*, 571-580.

Sauer, S., Kliem, M. 2010. Mass spectrometry tools for the classification and identification of bacteria. *Nat Rev Microbiol 8*, 74-82.

Schulthess, B., Bloemberg, G.V., Zbinden, A., Mouttet, F., Zbinden, R., Bottger, E.C., Hombach, M. 2016. Evaluation of the Bruker MALDI Biotyper for identification of fastidious gram-negative rods. *J Clin Microbiol 54*, 543-548.

Szacawa, E., Jawor, P., Dudek, K., Bednarek, D., Stefaniak, T. 2018. Screening for Mollicutes microorganisms in perinatal calf mortality cases in Polish dairy herds. *Pol J Vet Sci 21*, 441-444.

Thomas, A., Ball, H., Dizier, I., Trolin, A., Bell, C., Mainil, J., Linden, A. 2002. Isolation of mycoplasma species from the lower respiratory tract of healthy cattle and cattle with respiratory disease in Belgium. *Vet Rec 151*, 472-476.

Valentine, N., Wunschel, S., Wunschel, D., Petersen, C., Wahl, K. 2005. Effect of culture conditions on microorganism identification by matrix-assisted laser desorption ionization mass spectrometry. *Appl Environ Microbiol 71*, 58-64.

Welker, M., Moore, E.R. 2011. Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. *Syst Appl Microbiol 34*, 2-11.

Wise, K.S. 1993. Adaptive surface variation in mycoplasmas. *Trends Microbiol 1*, 59-63.

RAPID IDENTIFICATION OF *MYCOPLASMA BOVIS* FROM BOVINE BRONCHOALVEOLAR LAVAGE FLUID WITH MALDI-TOF MS AFTER ENRICHMENT PROCEDURE

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ABSTRACT

Mycoplasma bovis is a leading cause of pneumonia in modern calf rearing. Fast identification is essential to ensure appropriate antimicrobial therapy. Therefore, the objective of this study was to develop a protocol to identify *M. bovis* from bronchoalveolar lavage fluid (BALf) with MALDI-TOF MS and to determine diagnostic accuracy in comparison with other techniques. BALf was obtained from 104 cattle and presence of *M. bovis* was determined in three ways: (1) BALf was enriched and after 24, 48 and 72 hours of incubation analyzed by MALDI-TOF MS (RIMM), (2) triplex real-time PCR for *M. bovis*, *M. bovirhinis* and *M. dispar* and (3) ten day incubation on a selective-indicative agar. Diagnostic accuracy of the three tests was determined with Bayesian latent class modelling (BLCM). After 24h of enrichment, *M. bovis* was identified by MALDI-TOF MS in 3 out of 104 BALf samples. After, 48 and 72h of enrichment, 32/104 and 38/100 samples were *M. bovis* positive, respectively. Lipase positive Mycoplasma-like colonies were seen in 28 of 104 samples. Real-time PCR resulted in 28/104 positive and 12/104 doubtful results for *M. bovis*. The BLCM showed a sensitivity (Se) and specificity (Sp) of 86.6% (Confidence Interval: 69.4%-97.6%) and 86.4% (CI: 76.1-93.8) for RIMM. For real-time PCR Se was 94.8% (89.9-97.9) and Sp 88.9% (78.0-97.4). For selective-indicative agar, Se and Sp were 70.5% (52.1-87.1) and 93.9% (85.9-98.4), respectively. These results implicate that rapid identification of *M. bovis* with MALDI-TOF MS after an enrichment procedure is a promising test for routine diagnostics in veterinary laboratories.

Key words: Bayesian latent class model, lipase activity, *Mycoplasma bovis*, *Mycoplasma bovirhinis*, *Mycoplasma dispar*

INTRODUCTION

Mycoplasma bovis is one of the primary pathogens causing severe pneumonia in cattle, and is also associated with arthritis, otitis, mastitis and reproductive disorders (Maunsell and Donovan, 2009; Maunsell et al., 2001). Bovine respiratory disease (BRD) is the leading cause of antimicrobial use in calves (Pardon et al., 2012; Lava et al., 2016), and *M. bovis* is involved in approximately 20-30% of pneumonia outbreaks in conventional dairy or beef calves, and almost 100% of the veal calf herds have been in contact with this bacterium (Pardon et al., 2011; Francoz et al., 2015; Pardon et al., 2020). Rapid diagnosis of *M. bovis* is of great importance to rationally use antimicrobials and limit economic losses, since *M. bovis* is inherently resistant against widely used antimicrobial agents and difficult to eradicate once chronically present (Maunsell and Donovan, 2009; Gautier-Bouchardon, 2018). In contrast to other *Mycoplasma* species, *M. bovis* can be cultured quite well, although it easily takes 5-10 days before culture results become available. Also, to obtain definite *Mycoplasma* spp. identification, other techniques, such as biochemical characterisation or PCR are needed (Calcutt et al., 2018; Parker et al., 2018). This is of great importance, as *M. bovis* is generally recognised as a primary pathogen. However, pathogenic significance of other species such as *M. arginini*, *M. bovirhinis*, *M. dispar* are more controversial as they are both isolated from healthy and pneumonic lungs (Thomas et al., 2002; Bottinelli et al., 2017; Nicola et al., 2017; Timsit et al., 2018), and wrong identification could lead to unnecessary antimicrobial use. Selective-indicative agar using lipase activity as *M. bovis*-specific feature has been described to distinguish *M. bovis* from other bovine *Mycoplasma* spp. (Shimizu et al., 1983; Devriese and Haesebrouck, 1991). Unfortunately, its diagnostic performance is currently not known. PCR is the preferred method for final identification of *Mycoplasma* species. We currently observe a shift towards PCR identification directly on the specimen, such as bronchoalveolar lavage fluid (BALf) in case of pneumonia. Even though this is more rapid, due to logistic reasons laboratories usually collect samples to perform on a (two)-weekly analysis, whereupon diagnostic results still take several days.

MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry) is widely used as a rapid, low cost, culture-based diagnostic tool for identification of bacteria, including *Mycoplasma* spp. (Spergser et al., 2019). Nevertheless, prior isolation of *M. bovis* on specific solid media is still necessary and final identification can take up to ten days (Pereyre et al., 2013; Parker et al., 2018; Bokma et al., 2019). To reduce sample turnaround time, at present there is great interest in identification of bacteria by

MALDI-TOF MS directly from the sample or after a short enrichment period in liquid broth, as already done for urine, blood, milk or BALf specimens (Ferreira et al., 2011; Lallemand et al., 2017; Barreiro et al., 2018). However, such a technique is currently not available for *Mycoplasma* spp., presumably because of difficulties like their fastidious growth and overgrowth by other bacteria.

Therefore, the objective of this study was to develop a protocol to identify *M. bovis* directly from BALf after an enrichment procedure with MALDI-TOF MS. The accuracy of this diagnostic test was compared with real-time PCR and biochemical characterisation (lipase activity) on solid media in a Bayesian Latent Class Model (BLCM).

MATERIALS AND METHODS

Development of a protocol for fast MALDI-TOF detection of *M. bovis* in BALf

Determination of an enrichment procedure and antimicrobial concentrations

M. bovis concentration in BALf usually ranges from $1.8x10^3$ to $1.03x10^8$ CFU/ml (Castillo-Alcala et al., 2012), whereas a minimum concentration of $1.0x10^8$ CFU/ml is necessary to obtain interpretable spectra with MALDI-TOF MS starting from mycoplasma grown in broth (Pereyre et al., 2013; Bokma et al., 2019). Therefore, an enrichment procedure seemed necessary to identify *M. bovis* directly from BALf. We explored different broths as described earlier (Bokma et al., 2019) and experimented additionally with different antimicrobials, since overgrowth of *M. bovis* by other bacteria, such as (faecal) contaminants (eg. *Enterobacteriacae*), commensals (eg. *Streptococcus, Staphylococcus, Enterococcus*) or pathogenic bacteria (eg. *Pasteurellaceae*) in liquid media is very likely (Nicola et al., 2017; Sung et al., 2018).

Starting from a fresh culture, three *M. bovis* strains obtained from clinical field samples (Mb144, K6, K7; passaged 3-5 times) were cultured in modified basic PPLO-broth (#255420 BD Difco™, Berkshire, United Kingdom) with inactivated horse serum (25%, Gibco™, Ireland) and technical yeast extract (0.7%, Bacto™, Belgium), supplemented with sodium pyruvate (0.5%, Sigma-Aldrich, Germany), ampicillin sodium salt (520µg/mL, Sigma-Aldrich, Germany) (Bokma et al., 2019), and with colistin sulfate (VMD, Belgium), at a concentration of 967 IU/ml, similar as described previously (Angulo et al., 2003; Yassin et al., 2012). One mL of the *M. bovis* cultures with either meropenem (USP Reference Standard, Sigma-Aldrich, Germany) or vancomycin (Vancomycin hydrochloride from *Streptomyces orientalis*, Sigma-Aldrich, Germany) in final concentrations of 32, 16, 8, 4, 2, 1, 0.5 and 0

µg/mL were prepared in Eppendorf tubes at a final *M. bovis* concentration of 1-3x10^4 CFU/ml (Kanci et al., 2017). After 48 hours of incubation (37°C, 5% CO_2) protein extraction was performed as described before (Pereyre et al., 2013). Antimicrobial concentrations where the MALDI-TOF MS identification score (ID-score) for *M. bovis* was ≥1.7 were considered to not inhibit successful identification (Pereyre et al., 2013; Randall et al., 2015). The highest antimicrobial concentration that did not inhibit successful *M. bovis* identification with MALDI-TOF MS was chosen for the rapid identification protocol.

Identification and enrichment protocol with MALDI-TOF MS from BALf

The final protocol is presented in the next paragraphs as part of the diagnostic test study, and will be referred to as "Rapid identification of *M. bovis* with MALDI-TOF MS" (RIMM).

Diagnostic test study

Study population and sampling method

A prospective diagnostic test accuracy assay was performed. To detect a difference in sensitivity of 0.90-0.70 with 80% power, a minimum of 103 samples was needed (Bujang and Adnan, 2016). Therefore, a convenience sample of 104 BALf was collected for diagnostic purposes as described before (Van Driessche et al., 2017). Samples were taken from 3 week to 4 year old cows originating from 10 different farms (5 beef (A-D, F), 3 dairy (E, G, H) and 2 veal (I, J)) in Flanders, Belgium, between January 2019 and May 2019. Subsequently, samples were stored at 4°C for three to twenty hours before culture-based methods (index-tests: RIMM and selective-indicative agar) were performed. All samples were stored (-20°C) before the reference test (real-time PCR) was performed blindly. All procedures were approved by the local ethical committee under approval number EC2019-1.

Rapid identification of *M. bovis* with MALDI-TOF MS (RIMM)

BALf was vigorously vortexed and 4 mL was inoculated in 8 mL modified basic PPLO-broth as described above, supplemented with 32 µg/ml vancomycin and 32 µg/ml meropenem. After 24, 48 and 72 hours of incubation (37°C, 5% CO_2), protein extraction was performed on 1 mL of the enriched BALf culture as described before and 1 µL of lysate was spotted in triplicate on target polished steel BC plates (Pereyre et al., 2013; Bokma et al., 2019). Spotted samples were air dried and covered with 1 µL alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Bremen, Germany). All samples were processed with an Autoflex III smartbeam MALDI-TOF MS, using FlexControl and MBT Compass software (Bruker

Daltonics, Bremen, Germany). External calibration and validation were performed by adding Bacterial Test Standard, as described by the manufacturer (Bruker Daltonics, Bremen, Germany). Negative controls were performed by adding 1 µL of the matrix only. The standard library (server version 4.1.90 PYTH) was extended with four in-house main spectra profiles (MSPs) for *M. bovis* as outlined before (Bokma et al., 2019) and extra MSPs of *M. bovirhinis* (NCTC 10118), *M. ovipneumoniae* (NCTC 10151) and *M. dispar* (NCTC 10125). Identification of *Mycoplasma* spp. was considered as reliable at species level when logarithmic score values were ≥1.7 as proposed in previous studies (Pereyre et al., 2013; Randall et al., 2015), whereas non-mycoplasmal bacteria were considered reliable at species level when score values were ≥2.0 and at genus level between ≥1.7 and <2.0 (Ferreira et al., 2011).

Selective-indicative agar

100 µL of tenfold dilutions of BALf was inoculated on an in-house modified PPLO-agar, containing Difco™ PPLO-agar (#241210), enriched with 25% inactivated horse serum (Gibco™), 0.7% technical yeast extract (Bacto™), 0.5% D-(+)-glucose monohydrate (Sigma-Aldrich), 520 µg/mL ampicillin sodium salt (Sigma-Aldrich), 967 IU/ml colistin sulfate (VMD) and 0.1% Tween-80 (Polysorbate 80, Sigma-Aldrich). Tween-80 was added to observe lipase activity as indicator for *M. bovis* (Shimizu et al., 1983; Devriese et al., 1991). After 1-10 days of incubation (37°C, 5% CO_2), presumptive mycoplasma colonies (fried egg morphology) were identified as *M. bovis* based on the presence of lipase activity, observed as an "oil-like" film surrounding the colonies, and were counted.

Triplex real-time PCR

A previously described triplex real-time PCR was chosen as reference test as this method showed comparable results with other PCR methods used for routine diagnostics to identify *M. bovis* from BALf (Cornelissen et al., 2017; Wisselink et al., 2019). The limit of detection was determined at 30 CFU/ml for *M. bovis* and *M. bovirhinis*, and 300 CFU/ml for *M. dispar* as described previously (Cornelissen et al., 2017). BALf samples were thawed before DNA extraction. After vortexing, 200 µL sample was used for DNA extraction with the MagNA Pure 96 Instrument (Roche) using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) for DNA extraction. Five µL extracted DNA was used for the triplex real-time PCR detecting *M. bovis*, *M. dispar* and *M. bovirhinis* as described before (Cornelissen et al., 2017). Fresh *M. bovis*, *M. dispar* and *M. bovirhinis* cultures from in-house reference strains were

used as internal control to monitor DNA extraction, as well as inhibition of the PCR reactions. Ct values were interpreted as positive (< 35), doubtful (35-40) or negative (> 40), as previously described (Wisselink et al., 2019).

Conventional bacterial culture for non-mycoplasmal bacteria

An essential part of the protocol was to avoid overgrowth of *M. bovis* by other pathogens. Therefore to quantify other fast growing non-mycoplasmal pathogens and contamination present in the BALf samples, 100 µl of tenfold dilutions of BALf was cultured on Columbia agar, supplemented with 5% sheep blood (blood agar; Oxoid, UK). Non-mycoplasmal bacteria were identified with MALDI-TOF MS after one day of incubation (37°C, 5% CO_2). Additionally, after 72 hours of enrichment in the BALf in modified PPLO medium, 50 µl was cultured on blood agar for 24 hours (37°C, 5% CO2) to check for residual presence of non-mycoplasmal species.

Statistical analysis

Crosstabulation

First, the diagnostic accuracy (sensitivity and specificity) of both the RIMM and the selective-indicative agar (index tests) were determined with real-time PCR as reference test (WinEpi, Zaragoza, Spain). BALf was considered positive for *M. bovis* when ID-score of MALDI-TOF MS was ≥ 1.7 (direct identification) after 72 hours of incubation, Ct score < 40 (real-time PCR) or when Mycoplasma-like colonies showed lipase activity (bacterial culture). For 4 BALs, no results for 72h of incubation were obtained (due to a practical problem), for these samples results of 48h were used to compare with real-time PCR and the selective-indicative agar.

Bayesian latent class modelling

Definition of outcome tested

Sensitivity and specificity of the real-time PCR are not 100% (Cornelissen et al., 2017; Wisselink et al., 2019). Therefore, and also because of issues with clinical interpretation (detection of small amount of non-viable pathogens), this test cannot truly be considered a gold standard. To account for this issue, a second statistical analysis was performed to determine the diagnostic accuracy of the three methods. Bayesian latent class models create their own probabilistic definition of the outcome studied, depending on what the tests actually detect. In this study PCR detects DNA, either from living or death bacteria. The selective-

indicative agar detects culturable bacteria with lipase activity. Culture enrichment combined with MALDI-TOF MS detects protein spectra from culturable bacteria. Therefore, in our judgement the three tests detect three distinguished parameters, and a model for three independent tests was built. In addition, the model for dependent tests (with both culture-based methods being dependent of each other), was built to compare independent and dependent outcomes.

Model development

In order to access the accuracy of the three tests: (1) real-time PCR (detection of DNA), (2) RIMM (detection of proteins) and (3) selective-indicative agar (detection of lipase activity) to detect the presence of *M. bovis*, a latent class model (1 population, 3 tests) was considered, with each test regarded independent from the others. The unknown parameters of interest were sensitivity and specificity of the three diagnostic tests and the prevalence of *M. bovis* in the study population. Once the likelihood of the process generating the data observation is described, which is in this case a multinomial probability distribution, the estimation of posterior densities can be obtained using the Bayes theorem which links the likelihood with the posterior distribution (inference). At this stage, prior information on any parameter in the likelihood can be added to obtain posterior densities of the different parameters using a Markov Chain Monte-Carlo algorithm (Gibbs sampling). The prior information is a way to narrow parameter uncertainty when previous scientific information is available. In terms of prevalence and Se/Sp of tests, the priors are modelled using beta distributions that are naturally bound from 0 to 1. Priors can be uninformative (any value same probability of happening) or informative (some values are more or less probable) (Branscum et al., 2005).

Prior distribution determination process

Prior information was derived from available literature and expert opinion. Both for RIMM as for the selective-indicative agar, no literature information was available. Previous work on *M. bovis* real-time PCR, estimated a Se of 95.2% (CI95: 76.1%-99.9%) and Sp of 73.9% (51.6%-89.8%) (Cornelissen et al., 2017). Also for the prevalence of *M. bovis* in the population, prior information was available (Pardon et al., 2020; Gille et al., 2018). This literature information was combined with the best guesses of experts in the field (first author and senior authors). The 5th percentiles were guessed at 90%, 95% and 50% for Se, Sp and prevalence, respectively. These values were used to determine the beta distribution parameters of the corresponding prior distribution using a free online beta distribution calculator (Epitools,

Sergeant, ESG, Ausvet Animal Health Services and Australian Biosecurity calculator, available at http://epitools.eu/content.php?page=home, Ausvet) resulting in beta (99.7, 6.19), beta (1, 1) and beta (6.28, 13.32) for Se, Sp and prevalence, respectively.

In total three models were run. A first model with all prior information on all parameters set at uninformative (Beta 1,1). A second model included informative priors on Se and Sp of real-time PCR. The third model included informative priors on *M. bovis* prevalence and of real-time PCR.

The parameters of interest were determined based on a sample from the posterior distribution using Gibbs sampling with the WinBUGS statistical freeware (version 1.4.3., MRC Biostastics unit, Cambridge, UK). Estimation of posterior densities and model assessment was done using recommended techniques (Kostoulas et al., 2017). A total number of 100,000 iterations were used for each model, after a burn in of 5000 iterations. Three chains were run, with different initial values. Posterior median and 2.5-97.5 credibility intervals (95% CI) were extracted for each parameter. Model convergence was checked by visual inspection of density and Gelman-Rubin plots. Plots of chain autocorrelation were inspected to investigate the need of thinning of chains.

For smaller datasets, informative prior elicitation can be a process that could potentially have an impact on posterior density. Therefore, a sensitivity analysis was performed, running alternative models with highly different prior specifications to the main model. It was inspected whether posterior estimates of these alternatives models were included in the 95% credibility intervals of the main model (Branscum et al., 2005).

RESULTS

Development of a protocol for fast MALDI-TOF detection of *M. bovis* in BALf

Determination of an enrichment procedure and antimicrobial concentrations

After 48 hours of incubation, *M. bovis* strains were identified by MALDI-TOF MS as *M. bovis* (ID-score ≥2.0) after protein extraction for all tested antimicrobial concentrations. Therefore, a concentration of 32 µg/mL for both meropenem and vancomycin was selected for further testing.

Diagnostic test study

Triplex real-time PCR resulted in 26.9% (28/104) positive BALf samples (Ct ≤ 35), 11.5% (12/104) were doubtful (35 < Ct < 40) and 61.5% (64/104) were negative (Ct ≥ 40 or no

detection) for *M. bovis*. For *M. bovirhinis* 79.8% (83/104) were positive, 11.5% (12/104) doubtful, and 8.7% (9/104) negative, for *M. dispar* this was 92.3% (96/104), 3.8% (4/104), and 3.8% (4/104), respectively (Supplementary Table 1).

After 24h of enrichment with the RIMM method, 2.9% (3/104) of the BALf samples were positive for *M. bovis*, and after 48 and 72 hours, 30.7% (32/104) and 38.0% (38/100), respectively. For 4 samples no results were obtained after 72 hours of incubation, because of a practical problem. ID-scores for *M. bovis* ranged between 1.74 and 2.65 and are shown in Supplementary Table 1. *M. bovirhinis* was identified after 24h (BALf no. 18, 23, 28, 60 and 95), 48h (BALf 26, 28, 29, 32, 35, 38, 43, 60, 64, 102) and 72h (BALf 60, 63, 66 and 88) with ID-scores between 1.70 and 1.88. In BALf no. 24, *M. ovipneumoniae* was identified after 48 and 72h of incubation (ID-score: 1.78 and 1.72). *M. dispar* was not detected with the RIMM method in any sample throughout the entire experiment.

Out of 104 samples, 28 (26.9%) showed lipase positive Mycoplasma-like colonies on the selective-indicative agar (Supplementary Table 1) ranging from $1.0x10^1$ to $4.6x10^4$ CFU/mL BALf (mean: $3.8x10^3$; median $3.3x10^2$). 18% (19/104) of the BALf samples did not show additional bacterial growth on blood agar plates, whereas 81.7% (85/104) did, among which were multiple pathogens and commensals (Supplementary Table 1). Despite the presence of these non-mycoplasmal bacteria in the original BALf samples, the selective enrichment phase did not allow non-mycoplasmal bacterial growth to an extent that was able to be detected by MALDI-TOF MS.

Residual contamination was checked after 72 hours of enrichment. There was no microbial growth on blood agar in 70% of the BALf samples after 24 hours of incubation (70/100). For the samples with microbial growth on blood agar MALDI-TOF MS identified *Candida* spp. (4/30), *Aspergillus fumigatus* (2/30), *Stenotrophomonas maltophilia* (1/30) and *Staphylococcus* spp. (1/30). In 23 out of 30 blood agars showing microbial growth, no identification was possible with MALDI-TOF MS, but macroscopic appearance suggested mainly fungal contaminants.

Table 1. 2x2 Contingency table for direct MALDI-TOF MS identification as index test compared to real-time PCR as reference test for identification of *Mycoplasma bovis* from BALf samples (n=104).

Test		Reference test (real-time PCR)		
		Positive	Negative	Total
MALDI-TOF MS	Positive	75% (30/40)	14% (9/64)	39
	Negative	25% (10/40)	86% (55/64)	65
	Total	40	64	104
Selective-indicative agar	Positive	60% (24/40)	6% (4/64)	28
	Negative	40% (16/40)	94% (60/64)	76
	Total	40	64	104

The 2x2 contingency tables of the RIMM method and the selective-indicative agar compared with real-time PCR as reference test are shown in Table 1. The sensitivity (Se) and specificity (Sp) for the RIMM compared to real-time PCR were 75.0% (Confidence Interval 95%: 61.6-88.4%) and 85.9% (CI95: 77.4%-94.5%), respectively. The selective-indicative agar showed a 60.0% (CI95: 44.8%-75.2%) Se and 93.8% (CI95: 87.8%-99.7%) Sp, compared to the real-time PCR test.

All latent class models converged and prior distributions and posterior summary statistics of each model are shown in Table 2. Parameters were particularly stabile between models, and the results obtained from the sensitivity analysis were highly robust to changes in the prior distributions. Model three included the most prior information and is therefore expected to be the most accurate one. Prevalence of *M. bovis* was 32.6% (23.5%-42.6%), which was comparable to the prior information added to the third model. In independent model 3, RIMM showed a Se and Sp of 86.6% (69.4%-97.6%) and 86.4% (76.1%-93.8%). Real-time PCR had a Se of 94.8% (89.9%-97.9%) and Sp of 88.9% (78.0%-97.4%). The selective-indicative agar had a Se and Sp of 70.5% (52.1%-87.1%) and 93.9% (85.9%-98.4%), respectively. No significant difference between the independent and dependent model were observed.

Table 2. Posterior median and 95% credible interval of three independent Bayesian latent class models for the prevalence of *M. bovis* (Prev), sensitivity (Se) and specificity (Sp) of the Rapid identification of *M. bovis* with MALDI-TOF MS method (RIMM), triplex real-time PCR (PCR) and selective-indicative agar (SIA) used to diagnose *M. bovis* from bronchoalveolar lavage fluid samples.

	Model 1		**Model 2**		**Model 3**	
	Prior densities	Posterior densities	Prior densities	Posterior densities	Prior densities	Posterior densities
Se_{pcr}	Beta (1,1)	93.5 (77.2-99.7)	Beta (99.7,6.19)	94.8 (89.8-97.8)	Beta (99.7,6.19)	94.8 (89.9-97.9)
Sp_{pcr}	Beta (1,1)	89.2 (78.1-97.8)	Beta (1,1)	89.1 (78.1-97.7)	Beta (1,1)	88.9 (78.0-97.4)
Se_{rimm}	Beta (1,1)	86.0 (68.3-97.5)	Beta (1,1)	86.3 (68.8-97.5)	Beta (1,1)	86.6 (69.4-97.6)
Sp_{rimm}	Beta (1,1)	86.8 (76.3-94.9)	Beta (1,1)	86.5 (76.2-93.9)	Beta (1,1)	86.4 (76.1-93.8)
Se_{sia}	Beta (1,1)	69.5 (50.8-86.7)	Beta (1,1)	70.2 (51.8-86.9)	Beta (1,1)	70.5 (52.1-87.1)
Sp_{sia}	Beta (1,1)	94.1 (86.0-98.8)	Beta (1,1)	94.0 (86.0-98.5)	Beta (1,1)	93.9 (85.9-98.4)
Prev	Beta (1,1)	33.7 (23.2-45.9)	Beta (1,1)	33.3 (23.1-44.6)	Beta (6.28,13.32)	32.6 (23.5-42.6)

Model 1: No informative priors

Model 2: Informative priors on prevalence and Se_{pcr} (mode 95%; 5^{th} percentile = 90%) and Sp_{pcr} (mode 74%; 5^{th} percentile = 95%)(30)

Model 3: Informative prior on Se_{pcr}, Sp_{pcr} and prevalence of *M. bovis* in BALf (mode 30%; 5^{th} percentile = 50%)(33)

DISCUSSION

The objective of the present study was to achieve a proof of concept for rapid identification of *M. bovis* from bovine BALf samples after enrichment with MALDI-TOF MS and to compare these with another culture-based method (selective-indicative agar) and a DNA-based reference test (real-time PCR). In this study we were able to identify *M. bovis* from the majority of BALf with RIMM within two days after incubation, and even more after three days. The current protocol was able to reduce relevant growth of non-mycoplasmal bacteria and non-pathogenic *Mycoplasma* spp. present in BALf, resulting in high sensitivity (86.6%; CI95: 69.4-97.6) and specificity (86.4%; 76.1-93.8) of this diagnostic test as determined by BLCM. Prior information of the BLCM was extracted from peer-reviewed journals and sensitivity analysis for robustness of all models were verified. These together, make the possibility of bias due to the best guesses of the experts in the field less likely.

False negatives of the RIMM method compared to real-time PCR can be explained by the livability of *M. bovis*. PCR detects DNA, while for culture-based methods, like the MALDI-TOF MS, bacteria need to be alive. Some of the sampled calves might have been treated with antimicrobials before obtaining BALf resulting in non-viable *M. bovis* in the lung, so only DNA could be detected. This would suggest that no active *M. bovis* infection is currently present and the clinical relevance of positive results from the real-time PCR might be argued, in contrast to culture-based methods (RIMM and selective-indicative agar). In addition, cross reaction with *M. agalactiae* has been described for the currently used real-time PCR (Cornelissen et al., 2017), while MALDI-TOF MS has been described to accurately distinguish *M. bovis* and *M. agalactiae* (Pereyre et al., 2013; Spergser et al., 2019). Both previous arguments are therefore more likely resulting in a false positive PCR result, rather than a false negative culture-based result and therefore current specificity of the RIMM might be underestimated.

Incoherence between a negative result for *M. bovis* with real-time PCR, but positive with the RIMM method, might be explained by the fact that the BALf volume used for RIMM was 4 mL, while this was only 200 µL for the real-time PCR reference test and 100 µL for biochemical identification (lipase activity) on agar. BALf contains mucus clumps and cells, which could lead to a heterogenic suspension of *M. bovis*. Although samples were vortexed vigorously, it is possible that certain parts of the BALf did not end up in the aliquot for real-time PCR. This could have caused a higher chance for isolation using the RIMM method compared with detection using real-time PCR or selective-indicative agar. The ability to

process larger volumes with the RIMM method from BALf without extra labor, is an advantage over both other tests (real-time PCR and selective-indicative agar) and might even increase sensitivity of culture-based methods. Considering that most clinical samples in this study contained 10^2-10^3 CFU/mL, the generation time of *M. bovis* (two hours in exponential phase) (Bürgi et al., 2018), and that the detection limit of the MALDI-TOF MS is 10^8 CFU/mL (Pereyre et al., 2013), detection of *M. bovis* from BALf after 48 hours of incubation can be expected and is in line with the observations. For samples in which *M. bovis* was detected after 72 hours of incubation at earliest, for example the presence of other pathogens, mucus composition, number of inflammatory cells or other antimicrobial substances might have influenced the *M. bovis* growth rate or MALDI-TOF MS identification efficacy.

Various antimicrobials were added to the modified PPLO broth, as high antimicrobial resistance levels against different antimicrobials were observed in bacteria isolated from cattle (Graveland et al., 2010; Catry et al., 2016; Hordijk et al., 2019). Meropenem was considered due to its broad spectrum and strong activity against Gram-negative bacteria, although this would probably not suppress all bacterial growth in BALf as for example methicillin-resistant *Staphylococcus aureus* (MRSA) show resistance against meropenem in humans, but is also a common pathogen in calves (Watanabe et al., 2001). Therefore, vancomycin was used as well. Until now only very low levels to no resistance is detected against this antibiotic in MRSA and *Enterococcus* strains obtained from cattle (Barlow et al., 2017; de Jong et al., 2019). It should be kept in mind that critically important antibiotics, even though used under laboratory circumstances, should be properly disposed of after use.

Even though the current method was able to suppress non-mycoplasmal bacterial growth, there is still room for improvement for the reduction of yeast and fungi growth. In five of the BALf samples with a false negative MALDI-TOF MS result compared to real-time PCR, fungal growth was observed and this might have caused interference with identification of *M. bovis* as fungal pigments can suppress the desorption process (Buskirk et al., 2011), or may lead to the presence of interfering peaks. Adding an antimycotic drug such as Amphotericin B, might help increase sensitivity of the rapid MALDI-TOF MS identification method (Arai et al., 1966).

The real-time PCR cannot be seen as ‘gold standard’ technique for several reasons. First, sensitivity and specificity are not 100% (Cornelissen et al., 2017). Secondly, studies concluded that sensitivity of culture was sometimes higher than real-time PCR assays (Castillo-Alcala et al., 2012; Wisselink et al., 2019), and in this study 11.5% of the real-time PCR results were doubtful, and therefore difficult to compare to the culture-based methods

where culture was either positive or negative. Therefore, a third test was included in this study to perform a BLCM, as is recommended when no gold standard is available (Kostoulas et al., 2017).

The selective-indicative agar using lipase activity to identify *M. bovis* is not widely used, and therefore somewhat controversial. Though national laboratories in Belgium already use this method for years, this study is the first to show its diagnostic accuracy. This method resulted in a moderate sensitivity (70.5%) on the one hand, but on the other hand a specificity (93.8%) that was even higher than real-time PCR (88.9%). It has been described that not all *M. bovis* strains show lipase activity, which could explain false negatives, whereas some other *Mycoplasma* spp. do possess this characteristic, which could result in false positives (Rottem and Razin, 1964). Nevertheless, current results show that this medium might be helpful in *M. bovis* screenings where low cost is necessary and less experienced staff is located. It could also be helpful in choosing relevant colonies on agar plates for subsequent identification methods, such as real-time PCR. In addition, it cannot be ruled out that the use of other selective/indicative agar media, commercially available or not, could have resulted in different sensitivity or specificity data.

Other *Mycoplasma* spp. were identified as well in the BALf. Real-time PCR showed that at least 79.8% of the BALf samples were positive for *M. bovirhinis* and 92.3% for *M. dispar*. However, only 15.4% were positive for *M. bovirhinis* using the RIMM method with MALDI-TOF MS after 72 hours of enrichment. Mixture of *M. bovis, M. bovirhinis* and *M. dispar* in BALf of cattle are common (Thomas et al., 2002). Real-time PCR might have overestimated the prevalence of *M. bovirhinis* and *M. dispar*, because *M. bovirhinis* PCR cross-reacts with *M. canis*, and *M. dispar* PCR cross-reacts with *Acholeplasma axanthum* and *M. alkalescens* (Cornelissen et al., 2017). However, cross reaction can probably not explain the large difference observed between the RIMM method and the real-time PCR. The enrichment medium used in this study seemed to preselect for *M. bovis* growth. *M. bovirhinis* and *M. dispar* are glucose fermenting while in the current medium only pyruvate was added as carbon source (Tully and Whitcomb, 1979). The latter is a great advantage in the diagnosis of *M. bovis*, as *M. bovirhinis* and *M. bovis* can both be identified with MALDI-TOF MS after two days of incubation (Spergser et al., 2019). We did however observe a shift in identification of *M. bovirhinis* where two samples were positive after 24h and negative after 48h. As the medium was not optimal for *M. bovirhinis*, one reason could be that the concentration of (viable) *M. bovirhinis* balanced around the detection limit of the MALDI-TOF MS. Another reason might be that after 24h the concentration of *M. bovis* became higher

than the concentration of *M. bovirhinis,* as Pereyre et al. (2013) confirmed that *M. bovis* was the only pathogen recognized by MALDI-TOF MS, when samples contained 2-3 *Mycoplasma* species. Irrelevant *M. dispar* growth is less of a concern in culture-based methods, as this is a fastidious grower and more difficult to isolate (Tully and Whitcomb, 1979). Identification of *M. ovipneumoniae* from bovine BALf was unexpected. However, a recent study showed this species to be abundantly present in bovine BALf as well (Klima et al., 2019). Clinical relevance in cattle is unknown, although *M. ovipneumoniae* infections can have serious consequences in small ruminants, such as pneumonia, decreases in lamb production and average daily gain (Lindström et al., 2018; Manlove et al., 2019). Even though the MALDI-TOF MS specificity for accurate *M. ovipneumoniae* detection is not described, Spergser et al. (2019) tested 19 *M. ovipneumoniae* clinical isolates against their own in-house library, which resulted in 100% identification with a score value ≥ 1.7 (Spergser et al., 2019).

MALDI-TOF MS already proved to be of assistance for the identification of human and veterinary mycoplasmas from culture (Pereyre et al., 2013; Randall et al., 2015; Spergser et al., 2019). In the future it might be a great opportunity to develop a rapid and specific diagnostic tool to identify other pathogenic *Mycoplasma* spp. from BALf as well (for example *M. ovipneumoniae* from small ruminants or *M. pneumoniae* from humans) and accelerate turnover time in pneumonia diagnostics.

The current study shows that identification of *M. bovis* from BALf with the RIMM method is possible within 48-72 hours after sampling. Compared to real-time PCR, RIMM is probably cheaper, the clinical relevance might be higher, and when desired, it holds the opportunity to perform additional susceptibility testing and strain typing (Van Driessche et al., 2018; Hata et al., 2019). Therefore, rapid identification of *M. bovis* with MALDI-TOF MS is a promising method for diagnosis of *M. bovis* in veterinary laboratories. Yet, it is necessary for laboratories using this approach for the detection of *M. bovis* from clinical samples, to fully validate or comprehensively qualify this method. The validation parameters should include accuracy, precision, linearity and range of measurement, specificity, limit of detection, limit of quantitation, and robustness.

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SUPPLEMENTARY DATA. Table 1. Overview of identification scores (ID) after 72 hours of incubation with MALDI-TOF MS after enrichment method for *Mycoplasma bovis (Mb),* Ct scores (Ct) of triplex real-time PCR for *Mb*, *M. bovirhinis (Mbr)*, *M. dispar (Md)*, selective-indicative agar of *M. bovis* (SIA) obtained from 104 BALf originating from cattle in Flanders, Belgium (2019). Also results of blood agar plates are shown (after 24 hours of incubation).

No.	Farm	ID *Mb*	Ct *Mb*	Ct *Mbr*	Ct *Md*	SIA *Mb*	Blood agar (24 hours) Identification with MALDI-TOF
1	A	-*	-	25,30	23,47	-	*Mannheimia haemolytica, Moraxella branhamella ovis*
2	A	-*	-	27,19	23,15	-	*M. haemolytica, Staphylococcus cohnii, Bacillus species, Corynebacterium spp., Streptococcus suis*
3	A	2,08*	21,66	24,98	23,00	+	*M. haemolytica, Aeromonas veronii, Bacillus spp., Staphylococccus sciuri, Bibersteinia trehalosi*
4	A	-*	36,46	31,56	26,66	-	*M. haemolytica, S. sciuri, Staphylococcus lentus, Neisseria perflava*
5	A	-	-	27,74	23,16	-	*Histophilus somni*
6	A	-	-	29,45	23,57	-	*B. trehalosi, Streptococcus hyovaginalis*
7	A	-	-	25,26	23,06	-	*M. haemolytica, B. trehalosi, H. somni, Escherichia coli*
8	A	-	-	26,87	22,59	-	*M. haemolytica, H. somni, M. ovis*
9	A	-	-	26,39	21,73	-	*M. haemolytica, B. trehalosi, Bacillus subtilis*
10	A	-	-	33,90	26,58	-	*M. haemolytica*
11	A	-	-	30,20	23,96	-	*B. trehalosi*
12	A	-	-	28,08	21,48	-	*H. somni*
13	A	-	-	33,17	32,11	-	*H. somni*
14	A	-	-	30,01	24,17	-	*M. haemolytica, B. trehalosi, H. somni*
15	A	-	-	27,38	26,21	-	*H. somni, Staphylococcus equorum*
16	A	-	-	22,99	22,53	+	*M. haemolytica, H. somni, M. ovis*
17	A	1,74	-	26,93	24,23	+	*M. haemolytica, Pasteurella multocida*
18	A	2,08	37,00	30,18	25,80	-	*M. haemolytica, B. trehalosi, Streptococcus hyovaginalis, N. subflava, Staphylococcus xylosus*
19	A	1,81	24,26	29,56	21,40	+	*Gallibacterium anatis, Bacillus pumilus, Lactobacillus salivarius, Pelistega europaea*
20	A	1,75	29,09	28,55	23,28	+	*M. haemolytica, Staphylococcus aureus*
21	A	-	-	30,46	23,74	-	*M. haemolytica, H. somni, S. equorum*
22	B	-	-	36,08	40,29	-	-
23	C	-	-	30,39	28,06	-	-
24	C	-	-	27,09	23,72	-	-
25	C	-	-	-	23,26	-	*S. suis,, B. subtilis*
26	D	-	-	33,12	24,53	-	*Acinetobacter towneri, Serratia liquefaciens, Aerococcus viridans*
27	D	-	-	26,43	20,73	-	*P. multocida, M. ovis*
28	D	-	-	29,08	29,22	-	*E. coli, N. subflava, S. suis*
29	D	-	-	26,41	27,27	-	*M. ovis, S. sciuri*
30	D	-	-	26,66	25,10	-	*S. xylosus, Neisseria flavescens, S. suis*
31	D	-	-	26,61	26,41	-	*P. multocida*
32	D	-	-	-	-	-	-
33	D	-	-	29,37	26,83	-	*M. haemolytica, P. multocida, M. ovis, S. suis*
34	D	-	-	29,79	25,43	-	*P. multocida, M. ovis*
35	D	-	-	35,07	33,39	-	*S. sciuri*
36	D	-	-	27,62	20,61	-	*M. ovis, Staphylococcus haemolyticus*
37	D	-	-	29,33	26,39	-	*G. anatis, S. suis*
38	D	-	-	28,47	28,38	-	*M. ovis, Staphylococcus spp.*
39	D	-	-	22,28	21,95	-	*M. ovis, Staphylococcus chromogenes*
40	E	2,33	-	34,44	32,70	-	*E. coli*
41	E	-	32,24	38,18	27,26	-	*Morganella morganii, Lysinibacillus fusiformis*
42	E	2,42	-	30,23	28,94	-	*L. fusifromis, Providencia rettgeri*
43	E	-	39,55	-	29,61	-	-
44	E	2,24	27,71	29,22	28,58	+	*M. haemolytica, E. coli*
45	E	2,41	31,53	36,07	36,08	+	*Pantoea agglomerans, Staphylococcus felurettii, B. subtilis*
46	E	1,96	36,51	31,85	23,17	+	*H. somni*
47	E	2,41	31,80	27,15	26,06	+	*M. haemolytica, H. somni, S. suis*
48	E	2,36	36,13	28,31	24,71	-	*M. haemolytica*
49	E	2,11	32,39	27,29	21,50	+	*P. multocida, B. pumilus, Streptococcus dysgalactiae*
50	E	2,46	38,98	38,46	31,94	-	-

No.	Farm	ID *Mb*	Ct *Mb*	Ct *Mbr*	Ct *Md*	Tween-80 *Mb*	Blood agar (24 hours) Identification with MALDI-TOF
51	F	-	-	-	23,54	-	*M. haemolytica, S. suis*
52	F	-	-	-	37,32	-	-
53	F	-	-	29,89	30,13	-	*Mannheimia varigena, bacillus licheniformis, B. pumilus, Streptococcus pluranimalium*
54	F	-	-	31,47	30,11	-	*B.licheniformis, B. pumilus, S. pluranimalium, E. coli*
55	F	-	-	35,47	28,30	-	*B. trehalosi, B. pumilus*
56	F	-	-	34,53	38,17	-	*B. licheniformis, B. pumilus, S. pluranimalium, S. chromogenes*
57	G	-	-	36,06	-	-	-
58	H	-	-	25,58	23,06	-	*Moraxella bovoculi*
59	H	-	-	26,31	21,07	-	*P. multocida, M. bovoculi*
60	H	-	-	28,55	27,10	-	*M. bovoculi, Streptococcus spp. Trueperella pyogenes*
61	H	-	-	26,71	24,68	-	*P. multocida, M. bovoculi S. suis*
62	H	-	-	27,21	21,85	-	*P. multocida, M. ovis, Streptococcus*
63	H	-	-	37,00	27,10	-	*S. equorum*
64	H	-	-	31,03	23,80	-	*Moraxella spp., Streptococcus spp.*
65	H	-	-	31,53	23,31	-	*M. ovis, Neisseria meningitidis*
66	H	-	-	-	28,72	-	-
67	H	-	-	-	27,32	-	*Acinetobacter wolfii*
68	I	2,65	27,93	29,52	28,82	+	-
69	I	-	32,42	25,72	24,63	+	*M. haemolytica, B. licheniformis*
70	I	2,26	33,65	29,13	22,80	+	*M. haemolytica*
71	I	-	28,14	27,04	23,10	+	*M. haemolytica, Neisseria spp.*
72	I	2,16	31,65	28,00	22,75	+	*M. haemolytica*
73	I	2,08	40,87	36,66	24,30	-	-
74	I	2,35	34,14	28,79	22,47	-	*M. haemolytica*
75	I	-	31,28	25,69	21,06	+	*M. haemolytica, P. multocida,Streptococcus spp.*
76	I	1,93	30,51	27,18	20,81	+	*M. haemolytica, Stenotrophomonas spp., Bacillus spp.*
77	I	2,06	40,13	29,82	24,29	-	*M. haemolytica*
78	I	2,44	34,91	32,04	25,57	-	*P. multocida*
79	I	2,28	29,90	28,96	25,38	+	*M. haemolytica*
80	I	1,93	-	27,91	23,65	-	*P. multocida*
81	I	2,04	31,83	27,29	23,41	+	*M. haemolytica, P. multocida*
82	I	2,29	30,81	29,32	26,20	+	*P. multocida*
83	I	2,48	23,29	28,19	25,99	+	*P. multocida*
84	I	-	-	39,01	28,54	-	*P. multocida*
85	J	1,92	38,87	25,54	22,48	-	*M. haemolytica, S. suis*
86	J	-	-	32,45	23,39	-	*M. haemolytica*
87	J	-	-	38,95	35,40	-	-
88	J	-	35,92	-	32,20	-	*M. varigena*
89	J	2,01	34,15	26,03	25,00	+	*M. haemolytica, Staphylococcus cohnii, E. coli, S. suis, Streptococcus ferus*
90	J	-	37,67	-	41,68	-	-
91	J	1,84	38,30	28,92	23,03	-	*P. multocida, Staphylococcus saprophyticus*
92	J	2,05	40,24	27,68	26,31	-	*B. trehalosi*
93	J	2,2	34,39	29,04	23,81	-	*M. haemolytica, T. pyogenes*
94	J	1,96	28,00	24,61	26,42	+	*M. haemolytica, Serratia rubidaea, Citrobacter amalonaticus*
95	J	2,24	31,56	24,71	27,02	+	*S. chromogenes*
96	J	1,89	31,52	27,73	22,01	+	-
97	J	-	-	28,73	23,09	-	-
98	J	-	34,76	33,59	31,76	-	*M. haemolytica, P. multocida*
99	J	-	41,34	26,02	23,16	+	*M. haemolytica*
100	J	-	-	28,55	21,59	+	-
101	J	2,51	-	35,07	31,20	-	*M. haemolytica, E. coli*
102	J	1,92	35,03	27,88	27,25	-	*Staphylococcus haemolyticus*
103	J	-	39,30	34,68	30,39	-	*P. multocida*
104	J	1,84	31,61	24,81	22,02	+	*M. haemolytica, Moraxella branhamella ovis*

***** = result based on ID after 48h of incubation; Ct values were interpreted as positive (< 35), doubtful (35-40) or negative (>40)(Wisselink et al., 2019).

REFERENCES

Angulo, A.F., Jacobs, M.V., van Damme, E.H., Akkermans, A.M., de Kruijff-Kroesen, I., Brugman, J. 2003. Colistin sulfate as a suitable substitute of thallium acetate in culture media intended for mycoplasma detection and culture. *Biologicals 31*, 161-163.

Arai, S., Yoshida, K., Izawa, A., Kumagai, K., Ishida, N. 1966. Effect of antibiotics on growth of *Mycoplasma* pneumonia. *Mac J Antibiot 19,* 118-20.

Barreiro, J.R., Gonçalves, J.L., Grenfell, R., Leite, R.F., Juliano, L., Santos, M.V. 2018. Direct identification of bovine mastitis pathogens by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in pre-incubated milk. *Brazilian J Microbiol 49*, 801-807.

Barlow, R.S., McMillan, K.E., Duffy, L.L., Fegan, N., Jordan, D., Mellor, G.E. 2017. Antimicrobial resistance status of *Enterococcus* from Australian cattle populations at slaughter. *PLoS One 12*, e0177728.

Bokma, J., Pardon, B., Van Driessche, L., Gille, L., Deprez, P., Haesebrouck, F., Boyen, F. 2019. Optimizing identification of *Mycoplasma bovis* by MALDI-TOF MS. *Res Vet Sci 125*, 185-188.

Bottinelli, M., Passamonti, F., Rampacci, E., Stefanetti, V., Pochiero, L., Coletti, M., Rueca, F., Hyatt, D.R., Schnee, C. 2017. DNA microarray assay and real-time PCR as useful tools for studying the respiratory tract *Mycoplasma* populations in young dairy calves. *J Med Microbiol 66*, 1342-1349.

Branscum, A.J., Gardner, I.A., Johnson, W.O. 2005. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. *Prev Vet Med 68*, 145-63.

Bujang, M.A., Adnan, T.H. 2016. Requirements for minimum sample size for sensitivity and specificity analysis. *J Clin Diagnostic Res 10*, YE01-YE06.

Bürgi, N., Josi, C., Bürki, S., Schweizer, M., Pilo, P. 2018. *Mycoplasma bovis* co-infection with bovine viral diarrhea virus in bovine macrophages. *Vet Res 49,* 2.

Buskirk, A.D., Hettick, J.M., Chipinda, I., Law, B.F., Siegel, P.D., Slaven, J.E., Green, B.J., Beezhold, D.H. 2011. Fungal pigments inhibit the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of darkly pigmented fungi. *Anal Biochem 411*, 122-8.

Calcutt, M.J., Lysnyansky, I., Sachse, K., Fox, L.K., Nicholas, R.A.J., Ayling, R.D. 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. *Transbound Emerg Dis 65,* 91-109.

Castillo-Alcala, F., Bateman, K.G., Cai, H.Y., Schott, C.R., Parker, L., Clark, M.E., McRaild, P., McDowall, R.M., Foster, R.A., Archambault, M., Caswell, J.L. 2012. Prevalence and genotype of *Mycoplasma bovis* in beef cattle after arrival at a feedlot. *Am J Vet Res 73,* 1932-1943.

Catry, B., Dewulf, J., Maes, D., Pardon, B., Callens, B., Vanrobaeys, M., Opsomer, G., De Kruif, A., Haesebrouck, F. 2016. Effect of antimicrobial consumption and production type on antibacterial resistance in the bovine respiratory and digestive tract. *PLoS One 11,* e0146488.

Cornelissen, J.B.W.J., de Bree, F.M., van der Wal, F.J., Kooi, E.A., Koene, M.G.J., Bossers, A., Smid, B., Antonis, A.F., Wisselink, H.J. 2017. *Mycoplasma* detection by triplex real-time PCR in bronchoalveolar lavage fluid from bovine respiratory disease complex cases. *BMC Vet Res 13,* 97

De Jong, A., Simjee, S., Rose, M., Moyaert, H., Garch, F. El, Youala, M., Butty, P., Haag-Diergarten, S., Klein, U., Pellet, T., Schiffer, G., Serreyn, P.J., Vila, T. 2019. Antimicrobial resistance monitoring in commensal enterococci from healthy cattle, pigs and chickens across Europe during 2004-14 (EASSA Study). *J Antimicrob Chemother 74*, 921-930.

Devriese, L.A., Haesebrouck, F. 1991. Antibiotic Susceptibility Testing of *Mycoplasma bovis* using Tween 80 Hydrolysis as an Indicator of Growth. *Zentralbl Veterinarmed B 38*, 781-783.

Francoz, D., Buczinski, S., Bélanger, A.M., Forté, G., Labrecque, O., Tremblay, D., Wellemans, V., Dubuc, J. 2015. Respiratory pathogens in Québec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. *J Vet Intern Med 29*, 381-7.

Ferreira, L., Sánchez-Juanes, F., Muñoz-Bellido, J.L., González-Buitrago, J.M. 2011. Rapid method for direct identification of bacteria in urine and blood culture samples by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: Intact cell vs. extraction method. *Clin Microbiol Infect 17*, 1007-12.

Gautier-Bouchardon, A.V. 2018. Antimicrobial Resistance in *Mycoplasma* spp. *Microbiol Spectr. 6(4).*

Gille, L., Callens, J., Supré, K., Boyen, F., Haesebrouck, F., Van Driessche, L., van Leenen, K., Deprez, P., Pardon, B. 2018. Use of a breeding bull and absence of a calving pen as risk factors for the presence of *Mycoplasma bovis* in dairy herds. *J Dairy Sci 101*, 8284-8290.

Graveland, H., Wagenaar, J.A., Heesterbeek, H., Mevius, D., van Duijkeren, E., Heederik, D. 2010. Methicillin resistant *staphylococcus aureus* ST398 in veal calf farming: Human MRSA carriage related with animal antimicrobial usage and farm hygiene. *PLoS One 5*, e10990.

Gumpert, J. 2007. J. G. Tully and R. F. Whitcomb (Editors), The Mycoplasmas, Vol. II: Human and Animal Mycoplasmas. 509 S., 18 Abb., 30 Tab. New York-San Francisco-London 1979. Academic Press. *Z Allg Mikrobiol.*

Hata, E., Harada, T., Itoh, M. 2019. Relationship between antimicrobial susceptibility and multilocus sequence type of *Mycoplasma bovis* isolates and development of a method for rapid detection of point mutations involved in decreased susceptibility to macrolides, lincosamides, tetracyclines,. *Appl Environ Microbiol 85*, e00575-19.

Hordijk, J., Fischer, E.A.J., Van Werven, T., Sietsma, S., Van Gompel, L., Timmerman, A.J., Spaninks, M.P., Heederik, D.J.J., Nielen, M., Wagenaar, J.A., Stegeman, A. 2019. Dynamics

of faecal shedding of ESBL- or AmpC-producing *Escherichia coli* on dairy farms. *J Antimicrob Chemother 74*, 1531-1538.

Kanci, A., Wawegama, N.K., Marenda, M.S., Mansell, P.D., Browning, G.F., Markham, P.F. 2017. Reproduction of respiratory mycoplasmosis in calves by exposure to an aerosolised culture of *Mycoplasma bovis. Vet Microbiol 210*, 167-173.

Klima, C.L., Holman, D.B., Ralston, B.J., Stanford, K., Zaheer, R., Alexander, T.W., McAllister, T.A. 2019. Lower respiratory tract microbiome and resistome of Bovine Respiratory Disease mortalities. *Microb Ecol 78*, 446-456.

Kostoulas, P., Nielsen, S.S., Branscum, A.J., Johnson, W.O., Dendukuri, N., Dhand, N.K., Toft, N., Gardner, I.A. 2017. STARD-BLCM: Standards for the reporting of diagnostic accuracy studies that use Bayesian Latent Class Models. *Prev Vet Med 138*, 37-47.

Lallemand, E., Arvieux, C., Coiffier, G., Polard, J.L., Albert, J.D., Guggenbuhl, P., Jolivet-Gougeon, A. 2017. Use of MALDI-TOF mass spectrometry after liquid enrichment (BD BactecTM) for rapid diagnosis of bone and joint infections. *Res Microbiol 168*, 122-129.

Lava, M., Schüpbach-Regula, G., Steiner, A., Meylan, M. 2016. Antimicrobial drug use and risk factors associated with treatment incidence and mortality in Swiss veal calves reared under improved welfare conditions. *Prev Vet Med 126*, 121-130.

Lindström, L., Tauni, F.A., Vargmar, K. 2018. Bronchopneumonia in Swedish lambs: A study of pathological changes and bacteriological agents. *Acta Vet Scand 60*, 54.

Manlove, K., Branan, M., Baker, K., Bradway, D., Cassirer, E.F., Marshall, K.L., Miller, R.S., Sweeney, S., Cross, P.C., Besser, T.E. 2019. Risk factors and productivity losses associated with *Mycoplasma ovipneumoniae* infection in United States domestic sheep operations. *Prev Vet Med 168*, 30-38.

Maunsell, F.P., Donovan, G.A. 2009. *Mycoplasma bovis* Infections in Young Calves. *Vet Clin North Am Food Anim Pract 25*, 139-177.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle. *J Vet Intern Med 25*, 772-783.

Nicola, I., Cerutti, F., Grego, E., Bertone, I., Gianella, P., D'Angelo, A., Peletto, S., Bellino, C. 2017. Characterization of the upper and lower respiratory tract microbiota in Piedmontese calves. *Microbiome 5*, 152.

Pardon, B., De Bleecker, K., Dewulf, J., Callens, J., Boyen, F., Catry, B., Deprez, P. 2011. Prevalence of respiratory pathogens in diseased, non-vaccinated, routinely medicated veal calves. *Vet Rec 169,* 278.

Pardon, B., Catry, B., Dewulf, J., Persoons, D., Hostens, M., De Bleecker, K., Deprez, P. 2012. Prospective study on quantitative and qualitative antimicrobial and anti-inflammatory drug use in white veal calves. *J Antimicrob Chemother 67*, 1027-38.

Pardon, B., Callens, J., Maris, J., Allais, L., Van Praet, W., Deprez, P., Ribbens, S. 2020. Pathogen-

specific risk factors in acute outbreaks of respiratory disease in calves. *J Dairy Sci 103,* 2556-2566.

Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House, J.K. 2018. A review of mycoplasma diagnostics in cattle. *J Vet Intern Med 32*, 1241-1252.

Pereyre, S., Tardy, F., Renaudin, H., Cauvin, E., Del Prá Netto Machado, L., Tricot, A., Benoit, F., Treilles, M., Bébéar, C. 2013. Identification and subtyping of clinically relevant human and ruminant mycoplasmas by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol 51*, 3314-3323.

Randall, L.P., Lemma, F., Koylass, M., Rogers, J., Ayling, R.D., Worth, D., Klita, M., Steventon, A., Line, K., Wragg, P., Muchowski, J., Kostrzewa, M., Whatmore, A.M. 2015. Evaluation of MALDI-ToF as a method for the identification of bacteria in the veterinary diagnostic laboratory. *Res Vet Sci 101*, 42-49.

Rottem, S., Razin, S. 1964. Lipase activity of Mycoplasma. *J Gen Microbiol 37*, 123-34.

Shimizu, T. 1983. Selective medium for the isolation of *Mycoplasma bovis* from nasal discharges of pneumonic calves. *Res Vet Sci 34*, 371-3.

Spergser, J., Hess, C., Loncaric, I., Ramírez, A.S. 2019. Matrix-assisted laser desorption ionization-time of flight mass spectrometry is a superior diagnostic tool for the identification and differentiation of mycoplasmas isolated from animals. *J Clin Microbiol 57,* e00316-19.

Sung, J.Y., Hwang, Y., Shin, M.H., Park, M.S., Lee, S.H., Yong, D., Lee, K. 2018. Utility of conventional culture and MALDI-TOF MS for identification of microbial communities in bronchoalveolar lavage fluid in comparison with the GS Junior next generation sequencing system. *Ann Lab Med 38*, 110-118.

Thomas, A., Ball, H., Dizier, I., Trolin, A., Bell, C., Mainil, J., Linden, A. 2002. Isolation of *Mycoplasma* species from the lower respiratory tract of healthy cattle and cattle with respiratory disease in Belgium. *Vet Rec 151*, 472-476.

Timsit, E., Workentine, M., van der Meer, F., Alexander, T. 2018. Distinct bacterial metacommunities inhabit the upper and lower respiratory tracts of healthy feedlot cattle and those diagnosed with bronchopneumonia. *Vet Microbiol 221*, 105-113.

Van Driessche, L., Valgaeren, B.R., Gille, L., Boyen, F., Ducatelle, R., Haesebrouck, F., Deprez, P., Pardon, B. 2017. A Deep Nasopharyngeal Swab Versus Nonendoscopic Bronchoalveolar Lavage for Isolation of Bacterial Pathogens from Preweaned Calves With Respiratory Disease. *J Vet Intern Med 31*, 946-953.

Van Driessche, L., Bokma, J., Gille, L., Ceyssens, P.J., Sparbier, K., Haesebrouck, F., Deprez, P., Boyen, F., Pardon, B. 2018. Rapid detection of tetracycline resistance in bovine *Pasteurella multocida* isolates by MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA). *Sci Rep 8*, 13599.

Van Driessche, L., Bokma, J., Deprez, P., Haesebrouck, F., Boyen, F., Pardon, B. 2019. Rapid identification of respiratory bacterial pathogens from bronchoalveolar lavage fluid in cattle by MALDI-TOF MS. *Sci Rep 9,* 18381.

Watanabe, A., Tokue, Y., Takahashi, H., Kikuchi, T., Kobayashi, T., Gomi, K., Fujimura, S., Yasui, S., Murayama, Y., Nukiwa, T. 2001. Comparative in-vitro activity of carbapenem antibiotics against respiratory pathogens isolated between 1999 and 2000. *J Infect Chemother 7*, 267-71.

Wisselink, H.J., Smid, B., Plater, J., Ridley, A., Andersson, A.M., Aspán, A., Pohjanvirta ,T., Vähänikkilä, N., Larsen, H., Høgberg, J., Colin, A., Tardy F. 2019. A European interlaboratory trial to evaluate the performance of different PCR methods for *Mycoplasma bovis* diagnosis. *BMC Vet Res 15*, 86.

Yassin, M.H., Al-Humiany, A.A., Mansour, A.M.A. 2012. Colistin sulphate as a substitute of thallium acetate in culture media for isolation of Mycoplasma from cattle diseased by Mycoplasmosis. *Int J Microbiol 3*, 128-132.

DIAGNOSTIC TEST ACCURACY STUDY ON NANOPORE SEQUENCING FOR THE RAPID IDENTIFICATION OF *MYCOPLASMA BOVIS* FROM INDIVIDUAL AND POOLED RESPIRATORY TRACT SAMPLES

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ABSTRACT

Rapid identification of *Mycoplasma bovis* infections in cattle is a key factor to guide antimicrobial therapy and biosecurity measures. Recently, nanopore sequencing became an affordable diagnostic tool for both clinically relevant viruses and bacteria, including *Mycoplasma*, but the diagnostic accuracy for *M. bovis* identification is currently undocumented. Therefore, in this study nanopore sequencing was compared to rapid identification of *M. bovis* with MALDI-TOF MS (RIMM), and triplex real-time PCR in a Bayesian latent class model (BLCM) for *M. bovis* infection. Bronchoalveolar lavage fluid (BALf) was obtained from individual calves and analyzed with the three above mentioned methods. In practice, pooling of samples is often recommended. Therefore, a convenience sample of 17 pooled samples containing 5 individual BALf samples per farm was analyzed as well. The results of the pooled samples were compared to the individual samples, to determine sensitivity (Se) and specificity (Sp). The BLCM showed a good Se (77.4%; 95% Credible Interval: 58.6%-92.3%) and high Sp (97.3%; 91.1%-99.7%) for nanopore sequencing compared to RIMM (Se: 93.3%; 77.6%-99.5%, Sp: 92.7; 84.0%-98.0%) and real-time PCR (Se: 93.9%; 88.8%-97.3%, Sp: 86.0%; 76.0-93.6%). Se and Sp of pooled analysis for *M. bovis* were 68.8% (95% confidence interval: 52.7-84.8%) and 97.1% (93.0%-101.1%) for nanopore sequencing and 81.3% (67.7%-94.8%) and 85.3% (76.9%-93.7%) for RIMM, respectively. In conclusion, nanopore sequencing is a rapid and reliable tool for the identification of *M. bovis*. To reduce costs and increase chance of pathogen identification, pooling of 5 samples for nanopore sequencing and RIMM is possible.

Key words: Bayesian latent class model, bronchoalveolar lavage, MALDI-TOF MS, *Mycoplasma* species, selective indicative agar

INTRODUCTION

Many bovine *Mycoplasma* species can be isolated from cattle, but only few of them are considered pathogenic, in particular *Mycoplasma bovis* (Maunsell and Donovan, 2009; Caswell et al., 2010; Oliveira et al., 2020). Worldwide, *M. bovis* is associated with a long list of diseases, predominantly pneumonia and otitis in calves and mastitis and arthritis in adult cattle (Maunsell and Donovan, 2009; Maunsell et al., 2011). Early recognition of an *M. bovis* infection is crucial for different reasons. First, *M. bovis* is highly contagious and any delay in therapy increases the number of infected animals in a herd. Second, *M. bovis* is inherently resistant against many antimicrobials recommended in antimicrobial guidelines as empiric first intention treatment in several European countries, like beta-lactam antibiotics and sulphonamides (Lysnyansky and Ayling, 2016; Gautier-Bouchardon, 2018). Third, *M. bovis* has several virulence factors facilitating chronic infections, making antimicrobial therapy less successful when initiated later in the course of disease (Razin and Hayflick, 2010; Perez-Casal, 2020). Fourth, given its highly contagious nature, antimicrobial group treatment with an appropriate antimicrobial is a preferred control mechanism for *M. bovis* infections in both feedlot and veal calf industry. Therefore, rapid identification is important in the framework of antimicrobial use, antimicrobial resistance, and animal welfare. However, currently available routine diagnostic tests either take over a week before results are available (*e.g.* culture techniques) or are expensive and have interpretative issues regarding infectious load and clinical relevance (*e.g.* PCR) (Parker et al., 2018). Recently, a new culture-based method to identify *M. bovis* with MALDI-TOF MS from bronchoalveolar lavage fluid (BALf) was developed, with a significant lower sampling-to-diagnostic-result turnaround time (TAT) of 2-3 days (Bokma et al., 2020a). An alternative method for rapid identification may be whole genome sequencing, with the advantage that also strain typing and antimicrobial resistance determination may be possible (Bokma et al., 2020b; Kinnear et al., 2020; Ledger et al., 2020; Yair et al., 2020). Thus far next- and third generation sequencing technologies were highly time consuming, expensive, and therefore mainly applied in research settings or specific areas in human medicine (*e.g.* cancer identification). Newer real-time sequencing approaches (*e.g.* Oxford Nanopore Technologies) revolutionized the sequencing field, becoming affordable, and having sequencing data readily available. This makes it a new tool that is now available in veterinary medicine for quick metagenomics routine diagnostics (Theuns et al., 2018). One of the advantages of nanopore sequencing over culture-based methods, is the possibility of simultaneously identifying different *Mycoplasma* species, viruses and bacteria (McCabe et al.,

2018; Theuns et al., 2018). However, there is no information available concerning the diagnostic accuracy of nanopore sequencing used for the identification of *Mycoplasma* species directly from BALf. Despite the fact that knowledge on viral involvement when facing a respiratory disease outbreak is very informative (*e.g.* for vaccination), knowing whether *M. bovis* is involved is most crucial for antimicrobial decision making, including the decision for group therapy. For this purpose, an accurate, but also affordable test is preferred. In the field, to reduce costs and enhance the probability for pathogen detection, pools of samples from 5 animals are often made (Pardon and Buczinski, 2020). Nevertheless, whether pooling influences the sensitivity (Se) and specificity (Sp) of our previously described MALDI-TOF MS protocol (Rapid Identification of *M. bovis* by MALDI-TOF MS, RIMM; Bokma et al., 2020a) or nanopore sequencing protocols was not explored yet.

Therefore, the first aim of this study was to determine the Se and Sp of *M. bovis* identification with nanopore sequencing compared to real-time PCR and RIMM on individual samples in a Bayesian Latent Class Model (BLCM). The second aim was to determine diagnostic accuracy of pooled samples compared to individual samples for RIMM and nanopore sequencing.

MATERIALS AND METHODS

Diagnostic test study

Study population and sampling method

A prospective diagnostic test accuracy assay was performed. In our previous diagnostic test accuracy experiment, executed under the same circumstance, it was shown that 100 BALf were sufficient for the determination of Se and Sp of diagnostic methods for the identification of *M. bovis* (Bokma et al., 2020a). Therefore, a convenience sample of 100 BALf was collected for diagnostic purposes as described before (Van Driessche et al., 2017) and as approved by the local ethical committee (EC2020-068). Samples were taken between November 2020 and December 2020 from 3-week to 8-month-old calves originating from 14 different farms located in Belgium, and one in the Netherlands. BALf was stored at 4°C for maximum 20 hours until separating the samples for the different diagnostic methods, reflecting the routine diagnostic workflow. Whenever possible, a pool of samples from 1 farm was made from 5 BALf samples by taking 800 µL from each sample resulting in 4 mL aliquots. Both index-tests (nanopore sequencing and RIMM) were performed immediately after splitting the sample up, while samples for the *M. bovis* reference test (real-time PCR) were first stored at -20°C, prior to blind testing.

Nanopore sequencing for *Mycoplasma species*

Four mL of BALf and BALf pool (even ratio) was purified by aspirating the fluid through a newly developed swab in connection to a 0.8 µm surfactant-free cellulose acetate filter and a syringe. This sampler facilitates on-site purification of diagnostic samples and purifies viruses and bacteria for rapid sequencing. For nanopore sequencing, an innovative platform at PathoSense was used, which relies on an *in-house* developed sampling-to-diagnostics-interpretation workflow (Theuns et al., 2018). In short, samples were spiked with an internal control (semi-quantification purpose) prior to pathogen pre-enrichment using a nuclease treatment in order to remove free nucleic acids and only diagnose clinically relevant species and not free non-infectious pathogen nucleic acids. Subsequently nucleic acids were extracted and used for sequencing library preparation, multiplexing 12 samples at a time. Next, the samples were subjected to third-generation sequencing using an R.9.4.1 Flow Cell and MinION sequencer. Final sequencing data was processed using an *in-house* bioinformatics pipeline to profile the metagenomic context of the samples, highlighting both viral and bacterial (incl. *Mycoplasma* species) agents. Samples were interpreted as positive, when ≥ 5 reads for *Mycoplasma* species were detected.

Rapid identification of *M. bovis* with MALDI-TOF MS (RIMM)

Four mL of BALf was vigorously vortexed and processed as described before (Bokma et al., 2020a). Briefly, BALf was added to modified PPLO broth including vancomyin (32 µg/ml) and meropenem (32 µg/ml). After 48 and 72 hours of incubation (37°C, 5% CO_2), protein extraction was performed on 1 mL of the enriched BALf culture. The protein extraction was slightly adjusted in comparison to our previous study, as we now omitted the ethanol step for quicker identification and clearer peak spectra (Spergser et al., 2019). Eventually, lysates were spotted, covered with matrix, and analyzed with Autoflex III smartbeam MALDI-TOF MS, using FlexControl and MBT Compass software (Bruker Daltonics, Bremen, Germany). The standard library (server version 4.1.100 PYTH) was extended with four *M. bovis* strains, *M. bovirhinis* (NCTC 10118), *M. ovipneumoniae* (NCTC 10151) and *M. dispar* (NCTC 10125) (Bokma et al., 2020a). Identification of *Mycoplasma* spp. was considered reliable at species level when logarithmic score values were ≥1.7 as proposed in previous studies (Pereyre et al., 2013; Randall et al., 2015), whereas non-mycoplasmal bacteria were considered reliable at species level when score values were ≥ 2.0 and at genus level between ≥ 1.7 and < 2.0 (Ferreira et al., 2011).

Triplex real-time PCR for *M. bovis*, *M. bovirhinis*, and *M. dispar*

A previously described triplex real-time PCR targeting the 16S rRNA gene was chosen as reference test as this method showed comparable results with other PCR methods used for routine diagnostics to identify *M. bovis* from BALf (Cornelissen et al., 2017; Wisselink et al., 2019). Compared to DGGE a Se/Sp of 95.2% (95%CI: 76.2-99.9)/73.9% (51.5-0.90), 87.5% (47.4-100.0)/38.9% (23.1-56.5), and 100% (100.0-100.0)/19.4% (8.19-36.0) for *M. bovis*, *M. bovirhinis*, and *M. dispar* were obtained, respectively. The limit of detection (LOD) was determined at 30 CFU/ml for *M. bovis* and *M. bovirhinis*, and 300 CFU/ml for *M. dispar* as described previously (Cornelissen et al., 2017). The workflow was described in our previous diagnostic accuracy study (Bokma et al., 2020a), but briefly, BALf samples were thawed and 200 µL sample was used for DNA extraction with the MagNA Pure 96 Instrument (Roche) using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) for DNA extraction. Five µL extracted DNA was used for the triplex real-time PCR detecting *M. bovis*, *M. dispar* and *M. bovirhinis* (Cornelissen et al., 2017). Fresh *M. bovis*, *M. dispar* and *M. bovirhinis* cultures from *in-house* reference strains were used as internal control to monitor DNA extraction, as well as inhibition of the PCR reactions. Samples were processed in two separate runs. Cq values were interpreted as positive (< 35) or negative (≥ 35).

Bacterial culture for *M. bovis*, non-mycoplasmal bacteria, and fungal growth

For *M. bovis* isolation, 100 µL of BALf was inoculated on a selective-indicative agar, an *in-house* modified PPLO-agar (Bokma et al., 2020a). After 1-7 days of incubation (37°C, 5% CO_2), presumptive *Mycoplasma* colonies were identified as *M. bovis* based on the presence of lipase activity, observed as an “oil-like” film surrounding the colonies. For non-mycoplasmal bacteria 50 µl of BALf was cultured on Columbia agar, supplemented with 5% sheep blood (blood agar; Oxoid, UK), and incubated for 1-2 days (37°C, 5% CO_2). After 48-72 hours of incubation of the RIMM method, 50 µl was deposed on Columbia agar or Sabouraud dextrose agar with penicillin and streptomycin, and evaluated after 24-72 hours of incubation to determine fungal growth (37°C, 5% CO_2), as this was suggested as a potential inhibitor of *M. bovis* identification before (Bokma et al., 2020a). Identification of all pathogens was performed with MALDI-TOF MS by the direct transfer method (Randall et al., 2015).

Statistical analyses

Crosstabulation

First, the diagnostic accuracy (Se and Sp) of *M. bovis* identification with both nanopore sequencing and the RIMM method (index tests) were determined with real-time PCR as reference test (WinEpi, Zaragoza, Spain) for individual samples. This was also done for *M. bovirhinis* and *M. dispar* identification with nanopore sequencing. Subsequently, also nanopore sequencing and real-time PCR were compared to RIMM as reference test for the identification of *M. bovis*, as no 'gold standard' is available for *M. bovis*. BALf was considered positive for *M. bovis* when ≥5 reads were observed (nanopore sequencing), the ID-score of MALDI-TOF MS was ≥1.7 after 48 and/or 72 hours of incubation (RIMM) or the Cq score < 35 (real-time PCR). The diagnostic accuracy was also determined for the identification of *M. bovis* from individual and pooled samples with nanopore sequencing and RIMM (WinEpi, Zaragoza, Spain). In this case individual samples were the reference test (positive when at least one sample contained *M. bovis*), while the pooled samples (5 individuals) were the index test.

Bayesian latent class modelling

Definition of outcome tested

The Se and Sp of the triplex real-time PCR and RIMM method are not 100% (Cornelissen et al., 2017; Bokma et al., 2020a). Therefore, none of them can be used as 'gold standard' for the determination of the diagnostic test accuracy of nanopore sequencing. To circumvent this problem, a Bayesian latent class model was built. The BLCM is based on a common latent class between the diagnostic tests, and does not provide prior 'superiority' of one method over another. Nevertheless, it is possible to give the prior information whether two methods are more comparable to each other than the third method by including covariates (conditional dependent model). In this study, nanopore sequencing detects DNA from intact *M. bovis*, RIMM detects proteins from viable *M. bovis*, and real-time PCR detects DNA, both from living and dead *M. bovis*. Based on the detection component, one can argue whether a conditional dependent (nanopore sequencing and real-time PCR) or independent model is more appropriate. Both models were worked out, and compared to each other by visual comparison of Se and Sp. In addition, the deviance information criterion (DIC) was inspected as parameter for model fit.

Model development and prior distribution determination

The model was developed similarly to the model as described before (Bokma et al., 2020a; Rijckaert et al., 2020), using a code derived from S. Buczinski (University of Montreal, Canada). In short, a latent class model was considered to assess the accuracy of the three tests (Se and Sp) for *M. bovis* identification, being: (1) nanopore sequencing (detection of DNA from intact organisms), (2) RIMM (detection of proteins), and (3) real-time PCR (detection of DNA, both living and dead), and prevalence of *M. bovis* in the study population. Prior information was derived from available literature and expert opinion as described before (Bokma et al., 2020a). We decided to use the same prior information (Se 95%, Sp 74% of real-time PCR and 30% prevalence) as these were in line with our findings in the previous analysis (Cornelissen et al., 2017; Gille et al., 2018; Pardon et al., 2020). Nevertheless, we increased the 5th percentile from 50% to 80% for prevalence of *M. bovis*, as this prevalence was confirmed in our previous study on a similar study population (Bokma et al., 2020a). Priors for the RIMM method obtained by our previous study were not included, as the protocol was changed. The priors were modelled using beta distribution parameters of the corresponding prior distributions (Epitools, Sergeant, ESG, Ausvet Animal Health Services and Australian Biosecurity calculator, available at https://epitools.ausvet.com.au/betaparamsone, Ausvet), resulting in Beta (99.7, 6.19), Beta (1,1) and Beta (1.54, 2.26) for Se, Sp and prevalence, respectively.

Model analysis

In total three models were run for the conditional dependent and independent test in WinBUGS statistical freeware (version 1.4.3., MRC Biostastics unit, Cambridge, UK) using Gibbs sampling. In the first model prior information on all parameters was set at uninformative (Beta 1,1). The second model included informative priors on prevalence of *M. bovis*, and the third model included informative priors on prevalence of *M. bovis*, Se and Sp of real-time PCR. For each model, three chains with varying initial values were run for 100,000 iterations, after a burn in of 5,000 iterations. Model convergence was checked by visual inspection of density and Gelman-Rubin plots. Plots of chain autocorrelation were inspected to investigate the need of thinning of chains. The posterior median and 95% credibility intervals (CI) were extracted for each parameter. An additional sensitivity analysis was performed, running alternative models with highly different prior specifications to the main model. It was inspected whether posterior estimates of these alternatives models were included in the 95% credible interval of the main model (Branscum et al., 2005).

Quantification of *M. bovis* infection by qPCR and nanopore sequencing

To be able to quantify the concentration of *M. bovis* in the BALf with qPCR and potentially with nanopore sequencing, a ten-fold dilution series of *M. bovis* was made in distilled water. Concentration of the different dilutions was checked before freezing by counting colonies on solid PPLO medium as described earlier (Bokma et al., 2019). Subsequently, the aliquots were frozen at -20°C until triplex real-time PCR was performed as described above. Then, obtained Cq values were compared with the ten-fold dilution series for correlation, and with the logarithmic scale of the number of reads obtained by nanopore sequencing. Correlations were inspected visually and gave a general impression whether (semi)quantification of *M. bovis* with nanopore sequencing would be possible or not. In order to confirm the quantitative aspect of qPCR and nanopore sequencing, raw nanopore reads were first normalized to the internal control of the test, after which the logarithm was plotted against raw Cq values.

RESULTS

With nanopore sequencing 24% (24/100) of the BALf samples were positive for *M. bovis* (≥ 5 reads), and 76% (76/100) were negative (none or < 5 reads) (Supplementary file 1). Next to *M. bovis*, other *Mycoplasma* species were identified with nanopore sequencing, such as *M. bovirhinis* (49% of the samples), *M. dispar* (91%), *Ureaplasma* species (11%), *M. arginini* (7%), *M. penetrans* (4%), and *M. wenyonii* (4%). Triplex real-time PCR resulted in 36% (36/100) positive BALf samples (Cq < 35), and 64% (64/100) were negative (Cq > 35 or no detection) for *M. bovis*. For *M. bovirhinis* 67% (67/100) were positive, and 33% (33/100) negative, for *M. dispar* this was 88% (88/100) and 12% (12/100), respectively (Supplementary file 1). After 48-72h of enrichment with the RIMM method, 32% (32/100) of the BALf samples were positive for *M. bovis*. ID-scores for *M. bovis* ranged between 1.79 and 2.38 (Supplementary file 1). *M. bovirhinis* was identified in 5% (5/100) of the samples with ID-scores ranging from 1.70 to 1.85. *M. dispar* was not detected with the RIMM method in any sample throughout the entire experiment.

Out of 100 samples, 29 (29%) showed lipase positive *Mycoplasma*-like colonies on the selective-indicative agar. In addition, 15% (15/100) of the BALf samples did not show additional bacterial growth on blood agar plates, whereas 85% (85/100) did, but none of the identified bacteria on blood agar were detected with the RIMM method. This supports the expectation that all bacteria were killed or suppressed by the supplementation of the different antimicrobials. Residual contamination of fungal growth was checked after 72 hours of

enrichment. Out of the sabouraud or blood agar plates checked for fungal growth, 19% (19/100) showed fungal growth, which was not further identified. In two BALf (AB8, AB13) *Aspergillus* species were identified with the RIMM method after 72u of incubation. No *M. bovis* was detected in these samples by nanopore sequencing, real-time PCR or the selective-indicative agar.

Diagnostic test study of *M. bovis*

The 2x2 contingency tables of nanopore sequencing and RIMM compared with real-time PCR as reference test are shown in Table 1. The Se and Sp for the identification of *M. bovis* with nanopore sequencing compared to real-time PCR were 58.3% (Confidence Interval 95%: 42.2-74.4%) and 95.3% (CI95: 90.1%-100.5%), respectively. The RIMM showed a 72.2% (CI95: 57.6%-86.9%) Se and 90.6% (CI95: 83.5%-97.8%) Sp, compared to the real-time PCR test. When comparing the identification of *M. bovis* with nanopore sequencing and real-time PCR to the RIMM (Table 2), the Se and Sp for nanopore sequencing were 68.8% (CI95: 52.7-84.8%) and 97.1% (CI95: 93.0%-101.1%), respectively. Real-time PCR showed a 81.3% (CI95: 67.7%-94.8%) Se and 85.3% (CI95: 76.9%-93.7%) Sp, compared to the RIMM.

Table 1. 2x2 Contingency table for nanopore sequencing and RIMM as index test compared to real-time PCR as reference test for identification of *Mycoplasma bovis* from BALf samples (n=100).

Test		Reference test (real-time PCR)		
		Positive	Negative	Total
Nanopore sequencing	Positive	58% (21/36)	5% (3/64)	24
	Negative	42% (15/36)	95% (61/64)	76
	Total	36	64	100
RIMM	Positive	72% (26/36)	9% (6/64)	32
	Negative	28% (10/36)	91% (58/64)	68
	Total	36	64	100

The Se of nanopore sequencing for *M. bovirhinis* identification compared to real-time PCR was 73.1% (95CI: 62.5-83.7) and Sp 93.9% (85.8-102.1). For *M. dispar* this was 100% (100.0-100.0) and 66.7% (40-93.3), respectively. The 2x2 contingency tables are shown in Table 3.

Table 2. 2x2 Contingency table for nanopore sequencing and real-time PCR as index test compared to RIMM as reference test for identification of *Mycoplasma bovis* from BALf samples (n=100).

Test		Reference test (RIMM)		
		Positive	Negative	Total
Nanopore sequencing	Positive	69% (22/32)	3% (2/68)	24
	Negative	31% (10/32)	97% (66/68)	76
	Total	32	68	100
Real-time PCR	Positive	81% (26/32)	15% (10/68)	36
	Negative	19% (6/32)	85% (58/68)	64
	Total	32	68	100

Table 3. 2x2 Contingency table for nanopore sequencing as index test compared to real-time PCR as reference test for identification of *Mycoplasma bovirhinis* and *Mycoplasma dispar* from BALf samples (n=100).

Test		Reference test (Real-time PCR)		
		Positive	Negative	Total
Nanopore sequencing *M. bovirhinis*	Positive	73% (49/67)	6% (2/33)	51
	Negative	27% (18/67)	94% (31/33)	49
	Total	67	33	100
Nanopore sequencing *M. dispar*	Positive	100% (88/88)	33% (4/12)	92
	Negative	0% (0/88)	67% (8/12)	8
	Total	88	12	100

Bayesian latent class modelling

All models converged and the prior and posterior densities of model 1 to 3 are shown in Table 3. Parameters were very stable between models, although adding Se of triplex real-time PCR to model 3, had some influence on the posterior distributions. However, adding informative priors is a way to narrow parameter uncertainty when previous scientific information is available, therefore model 3 is expected to be the most accurate one. The results obtained from the sensitivity analysis were highly robust to changes in the prior distribution, only with extreme unrealistic low Se values (20%) or high prevalence (90%) the prior distributions were subject to small deviations from the 95% credible interval of model 3.

The differences between the outcomes of the conditional independent and dependent model were insignificant for most parameters, but in the dependent model, the Se of nanopore sequencing and real-time PCR decreased notably (Table 4). The DIC values of both the models differed minimally (< 2), with the lowest DIC for the conditional independent model. Based on these findings, we decided that the covariate term of the dependent model did not

Table 4. Posterior median and 95% credible interval (CI) of three conditional independent and one dependent Bayesian latent class models for the prevalence of *M. bovis* (Prev), sensitivity (Se) and specificity (Sp) of the identification with nanopore sequencing (np), rapid identification of *M. bovis* with MALDI-TOF MS method (rimm), and triplex real-time PCR (pcr) used to diagnose *M. bovis* from bronchoalveolar lavage fluid samples.

	Model 1 (independent)		**Model 2 (independent)**		**Model 3**	**(independent)**	**(dependent)**
	Prior densities	Posterior densities	Prior densities	Posterior densities	Prior densities	Posterior densities	Posterior densities
Se_{pcr}	Beta (1,1)	88.5 (72.1-97.8)	Beta (1,1)	88.5 (72.1-97.8)	Beta (99.7, 6.19)	93.9 (88.8-97.3)	86.9 (69.7-97.4)
Sp_{pcr}	Beta (1,1)	86.0 (76.0-93.6)	Beta (1,1)	86.0 (76.0-93.5)	Beta (1,1)	86.0 (76.0-93.6)	84.8 (74.9-92.2)
Se_{rimm}	Beta (1,1)	93.0 (77.0-99.5)	Beta (1,1)	93.0 (77.2-99.5)	Beta (1,1)	93.3 (77.6-99.5)	94.8 (89.8-97.8)
Sp_{rimm}	Beta (1,1)	93.5 (84.6-99.0)	Beta (1,1)	93.5 (84.6-99.0)	Beta (1,1)	92.7 (84.0-98.0)	94.4 (85.1-99.6)
Se_{np}	Beta (1,1)	76.2 (56.8-91.8)	Beta (1,1)	76.2 (57.0-91.8)	Beta (1,1)	77.4 (58.6-92.3)	74.1 (55.7-90.6)
Sp_{np}	Beta (1,1)	97.5 (91.3-99.8)	Beta (1,1)	97.5 (91.4-99.8)	Beta (1,1)	97.3 (91.1-99.7)	97.2 (91.0-99.8)
Prev	Beta (1,1)	29.8 (20.5-40.5)	Beta (1.54, 2.26)	29.8 (20.6-40.3)	Beta (1.54, 2.26)	29.1 (20.1-39.3)	30.0 (20.7-40.4)

Model 1: No informative priors

Model 2: Informative prior on prevalence of *M. bovis* in BALf (mode 30%, 5th percentile = 80%) (Bokma et al., 2020a; Pardon et al., 2020)

Model 3: Informative priors on and Se_{pcr} (mode 95%; 5th percentile = 90%), Sp_{pcr} (mode 74%; 5th percentile = 95%), and prevalence (Cornelissen et al., 2017)

improve the fit of the model, and we continued the analysis with the conditional independent model. Therefore, prevalence of *M. bovis* was 29.1% (95% Credible Interval: 20.1%-39.3%), nanopore sequencing had a Se and Sp of 77.4% (CI: 58.6%-92.3%) and 97.3% (CI: 91.1%-99.7%), respectively. RIMM showed a Se and Sp of 93.3% (CI: 77.6%-99.5%) and 92.7 (CI: 84.0%-98.0%). The Se and Sp of real-time PCR were 93.9% (CI: 88.8%-97.3%) and 86.0% (CI: 76.0-93.6%), respectively.

Pooling of BALf for nanopore sequencing and RIMM

In total, 17 pools were analyzed for both nanopore sequencing and RIMM as described above. In Supplementary file 2, an overview is shown of the *M. bovis* identified in the individual and pooled samples by both nanopore sequencing and the RIMM method. In Table 5, the contingency table of both individual and pooled samples with nanopore sequencing and RIMM are shown. Se and Sp for the identification of *M. bovis* in pooled samples with nanopore sequencing are 85.7% (CI95: 59.8-111.6%) and 90.0% (CI95: 71.4-108.6%), respectively. While for the RIMM method a 100% (CI95: 100-100%) Se and 88.9% (CI95: 68.4-109.4%) Sp were obtained.

Table 5. 2x2 Contingency table for nanopore sequencing and RIMM comparing pooled samples with individual samples for identification of *Mycoplasma bovis* from BALf samples (n=17).

Test		Individual sample		
		Positive	Negative	Total
Pooled Nanopore sequencing	Positive	86% (6/7)	10% (1/10)	7
	Negative	14% (1/7)	90% (9/10)	10
	Total	7	10	17
Pooled RIMM	Positive	100% (8/8)	11% (1/9)	9
	Negative	0% (0/8)	89% (8/9)	8
	Total	8	9	17

Pooled samples were categorized as positive, when at least one out of the five individual sample was positive

Quantification of *M. bovis* infection by qPCR and nanopore sequencing

The tenfold dilution series showed a concentration range of approximately 8.0 x 10^7 to 8.0 x 10^1 CFU/mL. Between tenfold concentrations, the Cq scores differed with a mean of 3.80 (standard deviation: 0.78; range: 2.67-5.42). The calibration line is shown in Figure 1 (determination coefficient $R^2 = 0.995$). In Figure 2, the number of *M. bovis* reads obtained by

nanopore sequencing are outlined on a logarithmic scale in respect to the Cq-values obtained by real-time PCR for *M. bovis*, showing a good agreement ($R^2 = 0.87$) between both values.

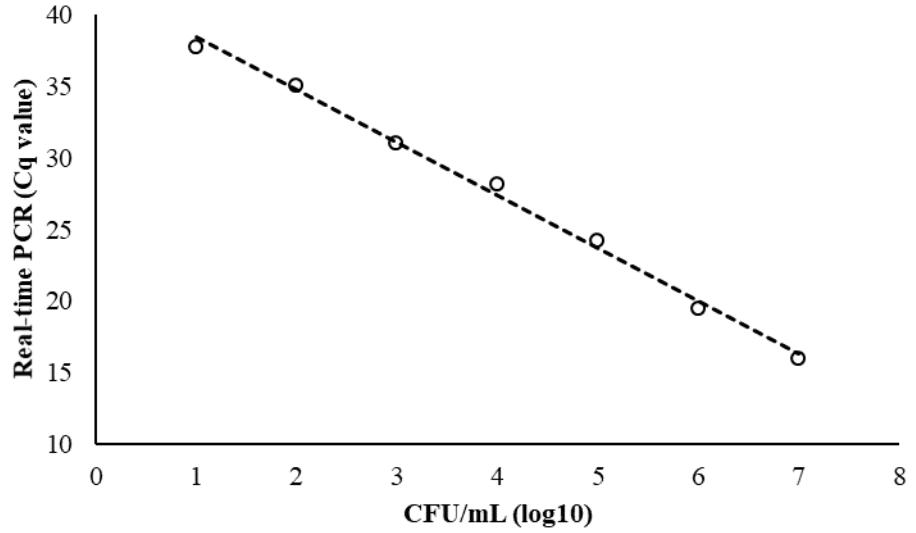

Figure 1. Calibration line of Cq values and logarithmic concentration of *M. bovis* in CFU/mL ($R^2 = 0.995$), showing the correlation between Cq value and *M. bovis* concentration in BALf samples as determined with colony counting.

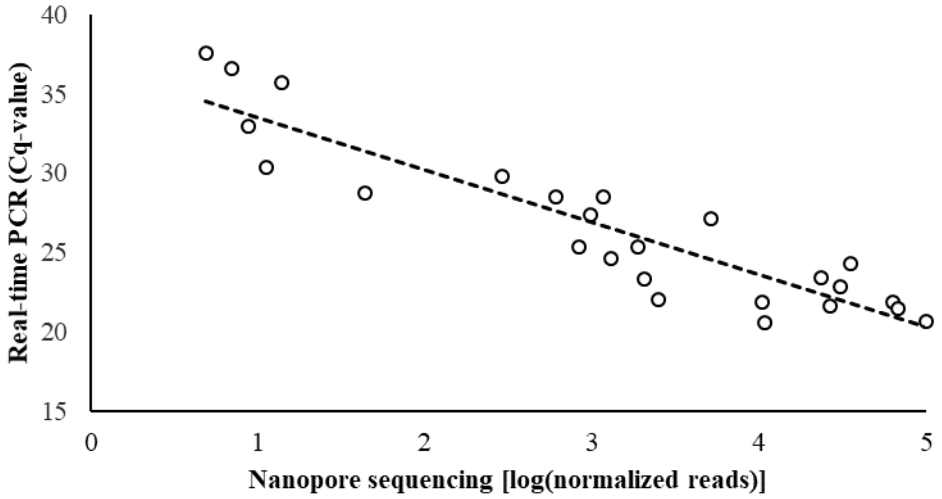

Figure 2. The logaritmic number of nanopore sequencing *M. bovis* reads and the Cq-values obtained by real-time PCR for *M. bovis* on 100 BALf from calves ($R^2 = 0.87$). BALf with 0 reads were excluded from the figure.

DISCUSSION

In this study, it was shown that nanopore sequencing can readily (24-36 hours turnaround time) identify *M. bovis* from BALf with reasonable Se (77.4%; CI 58.6-92.3) and high Sp (97.3%; CI 91.1-99.7) compared to identification with RIMM and real-time PCR. The disagreement between methods on individual samples can be addressed into different categories, being differences caused by (1) the protocol, (2) the component of interest and limit of detection, and (3) the interpretation of results.

Differences in start volumes and differences in sample preparation can explain the first category of disagreement. BALf can contain mucus clumps and cells, which could have led to a heterogenic suspension of *M. bovis*. However, as volumes between protocols were not equal (4 mL for nanopore sequencing/RIMM, 200 µL for real-time PCR), this could have been advantageous for methods using larger volumes. More detail on this subject is available elsewhere (Bokma et al., 2020a). Here, the preparation protocol of nanopore sequencing included an additional filter step to discard sample impurities. Potential biofilm and microcolony formation or clumping with other bacteria and mucus could have resulted in (partial) loss of *M. bovis* (McAuliffe et al., 2006; Hoelzle et al., 2020) during the filter step for nanopore sequencing or during splitting of the samples for real-time PCR.

The second category of disagreement (component of interest and limit of detection) also has its repercussions on the third category (interpretation). All three methods in the BLCM focus on the detection of different biological components of *M. bovis*. Current real-time PCR will detect DNA from culturable, viable but not culturable (those who can multiply) and dead *M. bovis*. Nanopore sequencing targets nucleic acids of clinically relevant intact (viable, both culturable and non-culturable) *M. bovis* and other pathogens due to the nuclease treatment, while RIMM can only detect culturable *M. bovis* as an enrichment procedure is necessary to exceed the detection limit of the MALDI-TOF MS. Therefore, real-time PCR is more sensitive to detect causative agents than culture-based methods when diseased animals already received antimicrobial therapy (Maurin, 2012). In farm ‘AB’, where disagreement between methods was observed, a previous *M. bovis* outbreak had been extensively treated with antimicrobials 4 to 6 weeks before sampling. Possibly only residual *M. bovis* DNA was left, which may explain the relatively high Cq values (>30) with real-time PCR and no identification of *M. bovis* with nanopore sequencing or RIMM (Supplementary file 1). In addition, this raises the question of clinical relevance of detecting elevated Cq values (>30). Another possibility of not detecting *M. bovis* with RIMM, could be the interference of fungal

growth at this farm (possibly induced by the intensive antimicrobial use), as 53% (8/15) of the samples showed fungal growth after incubation (in contrast to an overall percentage of only 19%), and interference has been described (Buskirk et al., 2011). In farm 'AI' the same incongruence was observed. In this farm *M. bovis* was previously diagnosed, calves were frequently treated with antimicrobials, and pregnant cows received an autogenous vaccine containing *M. bovis* intramuscularly twice (halfway gestation, and three weeks before partus). It has been described that autogenous vaccine can reduce colonization and spread of *M. bovis* (Nicholas et al., 2002; Dudek et al., 2016), but the role of passive immunity of the calves, coming from the mother is not clear (Maunsell et al., 2011). Therefore, in this farm it may be that no clinical infection was present anymore, or that very low numbers of *M. bovis* were present, not exceeding the LOD of nanopore sequencing and RIMM. The LOD of the real-time PCR (30 CFU/ml; Cornelissen et al., 2017) could have been lower than nanopore sequencing. Unfortunately, we did not define the LOD of nanopore sequencing, as we were not able to include the *M. bovis* ten-fold dilution series in the nanopore sequencing protocol at the time of preparing the dilutions. Freezing and thawing, as was done with real-time PCR, is not possible, as we observed that this can result in unreliable results for *M. bovis* identification with nanopore sequencing (unpublished results). We did however show, that (semi)quantification with nanopore sequencing is possible. To get better insights in the extent of this quantification, differentiation between culturable, viable, but not culturable, and dead *M. bovis* should be made in future studies.

The third category of disagreement related to the interpretation of different outcome values and relevance for clinical interpretation, is especially based on cut off values for positive *M. bovis* identification with nanopore sequencing (≥ 5 reads) and real-time PCR (Cq < 35). In BALf with Cq-values > 30 for *M. bovis*, no or very low reads were obtained by nanopore sequencing. This is not surprising, as the aim of the technology is only diagnosing viable pathogens (Theuns et al., 2018). Due to the use of a nuclease treatment in the nanopore protocol, non-infectious pathogen nucleic acids are discarded, whereas this is being detected in the real-time PCR flow. The latter should be taken carefully into account when interpreting real-time PCR results. This was also seen for the RIMM method, where in 6 out of 8 negative BALf, real-time PCR results showed a Cq-value of 32 to 35, while only 3 of the negative RIMM BALf for *M. bovis* had a Cq value < 32. This could be related to a lower Se of the latter methods or the presence or absence of a clinical infection versus residue of *M. bovis* DNA, contamination of the sample or aspecific hits in the real-time PCR test. In a previous study it was shown that in veal calves, four weeks after antimicrobial treatment, 49% of the

nasal swab samples were still positive for *M. bovis* with real-time PCR (mean Cq = 30.4), while only 19% of the samples were positive with culture (Becker et al., 2020). At the moment of the outbreak, 51% of the samples were positive for *M. bovis* with real-time PCR (mean Cq = 25.7) and 52% with culture. It was suggested that animals that were positive on real-time PCR, but negative on culture were in the recovery phase (Becker et al., 2020). (Re)starting antimicrobial treatment in this phase would not be recommended. However, optimizing cut off values of real-time PCR taking into account clinical relevance should be considered carefully, as small adjustments can result in different outcomes, resulting in different treatment of animals with potentially great consequences for antimicrobial use, antimicrobial resistance, and animal welfare. Therefore, more research is necessary to determine the association between test outcomes and the clinical status of the calf. Nevertheless, even though clinical examination and thoracic ultrasound can aid in determining the clinical status of the animal, the degree of self-cure in *M. bovis* infections is not clear. Next to this, the combination of multiple pathogens in a respiratory outbreak may hamper clear associations between detected pathogens and results. Once these associations are more established, Cq values and nanopore sequencing output may be used to support the evaluation of antimicrobial treatment efficacy and prognosis in acute or chronic bacterial infections, which has already been proposed in human medicine for *Mycobacterium tuberculosis* infections (Broccolo et al., 2003; Takahashi et al., 2007). Also, in *Mycoplasma pneumonia* patients, higher bacterial concentrations detected by real-time PCR were associated with severe respiratory disease, showing its potential as follow up tool (Nilsson et al., 2010).

Pooling five BALf for the identification of *M. bovis* showed high Se and Sp for nanopore sequencing and RIMM compared to individual sample processing. Pooling of samples can reduce diagnostic costs and can result in a more efficient diagnostic workflow when a high turnaround of samples is required, for example when the complete herd needs to be screened for eradication programs (Murai et al., 2014; Lohse et al., 2020). Pooling can also increase the possibility of detecting the causative pathogen of the disease (Pardon and Buczinski, 2020). In this study, we had a convenience sample of 17 pooled BALf samples. Pooled RIMM analysis showed very high Se (100%; CI95: 100-100) and Sp (88.9%; CI95: 68.4-109.4) for the identification of *M. bovis*. The loss in Sp of the RIMM method, was probably caused by a 'false negative' in the reference test (the individual samples, AI8), as all other methods, including selective-indicative agar, were positive for this sample. It is likely that the Sp of pooled RIMM was even underestimated in this study. Pooling of five BALf for nanopore

sequencing, also showed good Se (85.7%; CI95: 59.8-111.6) and Sp (90.0%; CI95: 71.4-108.6%) for the identification of *M. bovis*. Dilution of samples resulting in concentrations below the LOD, interference of high numbers of other pathogens present in the samples or different volumes/parts of the sample, are the most likely reasons for the slight loss in Se and Sp by pooling. Nevertheless, pooling of samples for nanopore sequencing and RIMM seems very reliable, although research on a larger sample size and different populations is still recommended for further confirmation.

Besides the identification of *M. bovis*, also other *Mycoplasma* species were identified with nanopore sequencing and triplex real-time PCR. Nanopore sequencing showed good Se and Sp for *M. bovirhinis* and *M. dispar* compared to the triplex real-time PCR, although these results should be interpreted with caution, as this triplex real-time PCR test is far from being 'the gold standard' for these species. *M. bovirhinis* and *M. dispar* are frequently isolated from BALf (Thomas et al., 2002; Timsit et al., 2018; Bokma et al., 2020a), and also in the present study this was the case. In our previous study, we speculated that the high prevalence could be partly attributed to the cross-reactivity of the real-time PCR with *Acholeplasma axanthum*, *M. alkalescens* and *M. canis*, but none of these *Mycoplasma* species were detected with nanopore sequencing, making this hypothesis less likely. Nonetheless, cross-reactivity with *M. penetrans, M. arginini*, *Ureaplasma* species and *M. wenyonii*, which were detected with nanopore sequencing, were not included in the study of Cornelissen et al. (2017), and could still result in cross-reactivity issues. The detection of *M. penetrans* in calves, detected by nanopore sequencing is an extraordinary finding, as this bacterium is usually detected in the urogenital or respiratory tract of humans with the human immunodeficiency virus (HIV) (Grau et al., 1995; Preiswerk et al., 2020). *M. penetrans* has only been associated with respiratory disease in a non-HIV patient once (Yáñez et al., 1999), and together with the relatively low number of reads detected in bovine BALf, more research is necessary to clarify whether this identification was a false positive potentially caused by cross-reactivity (*e.g.* with other *Mycoplasma* species) or whether calves can truly carry *M. penetrans*. Another unexpected finding was the identification of *M. wenyonii* in BALf, as this pathogen is a hemoplasma mostly diagnosed from whole blood (Tagawa et al., 2008). *M. wenyonii* is usually subclinical, but can cause several clinical signs, such as limb edema, swollen teats, anemia and milk drop, but so far pneumonia was not described (Smith et al., 1990; Genova et al., 2011; Nouvel et al., 2019). During BALf sampling, sometimes small blood vessels are damaged, and indeed some of the BALf where *M. wenyonii* was identified from, contained visible traces of blood.

In conclusion, the current study showed nanopore sequencing as a reliable cost-efficient method for the rapid identification of *M. bovis* and certain other *Mycoplasma* species in BAL fluid of calves. To reduce the cost and increase the chance of identifying *M. bovis*, BALf can be pooled and analyzed with both nanopore sequencing or RIMM. The advantage of nanopore sequencing, is the simultaneous rapid detection of viruses (1-2 days), while the advantage of the RIMM is that an *M. bovis* isolate is rapidly obtained (2-3 days), and fast additional strain typing (Chapter 4; Bokma et al., 2020b) or antimicrobial susceptibility testing with nanopore sequencing can be performed (Chapter 5.2).

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CONFLICT OF INTEREST

Sebastiaan Theuns and Hans Nauwynck are co-founders of PathoSense BV, a spin-off company of Ghent University. Nick Vereecke is PhD student at PathoSense BV and Ghent University.

SUPPLEMENTARY DATA

Supplementary Table 1. Overview of individual test results for *Mycoplasma* species.

Supplementary Table 2. Overview of the identification of *Mycoplasma bovis* in individual BALf (n=85) and pooled samples (n=17) by nanopore sequencing and the RIMM method.

Supplementary Table 1. Overview of identification scores for *M. bovis* (ID *Mb*) after 48-72 hours of incubation with RIMM, Cq scores (Cq) of triplex real-time PCR for *Mb*, *M. bovirhinis (Mbr)*, *M. dispar (Md)*, reads with nanopore sequencing, and selective-indicative agar of *M. bovis* (SIA) obtained from 100 BALf originating from calves.

No	Farm + Nº	Reads *Mb*	ID *Mb*	Cq *Mb*	Reads *Mbr*	Cq *Mbr*	Reads *Md*	Cq *Md*	SIA *Mb*
1	AA1	-	-	-	72	29.40	18640	25.11	-
2	AA2	-	-	33.92	9	30.20	1978	25.72	-
3	AA3	-	-	-	24	28.04	552	26.27	-
4	AA4	-	-	-	73	27.34	2806	25.03	-
5	AA5	-	-	-	36	27.31	12535	22.89	-
6	AB1	-	-	-	-	37.64	3380	25.15	-
7	AB2	-	-	32.85	18	28.41	10359	22.50	-
8	AB3	-	-	-	-	33.07	141	26.29	-
9	AB4	-	-	-	-	36.03	681	30.06	-
10	AB5	-	-	-	6	26.18	717	24.01	-
11	AB6	-	-	-	-	-	823	27.05	-
12	AB7	-	-	-	1	-	1130	24.32	-
13	AB8	-	-	-	-	-	373	24.12	-
14	AB9	-	-	-	1418	22.71	1078	24.11	-
15	AB10	-	-	33.42	-	39.12	592	26.32	-
16	AB11	-	-	-	-	-	375	26.47	-
17	AB12	-	-	-	-	-	396	28.50	-
18	AB13	-	-	36.67	-	-	121	31.14	-
19	AB14	-	-	-	-	-	1115	24.48	-
20	AB15	-	-	-	3	25.81	315	22.22	-
21	AC1	-	-	-	9	28.33	365	26.29	-
22	AC2	-	-	-	1	32.98	5	34.90	-
23	AC3	-	-	39.65	7	27.18	503	25.71	-
24	AD1	1	-	36.62	432	25.66	1937	25.48	-
25	AD2	1540	1.96	20.57	1016	23.22	40098	19.12	+
26	AD3	1	1.91	35.72	110	26.57	1567	24.70	-
27	AD4	1	-	32.97	3	28.79	66	30.34	-
28	AD5	41	1.81	24.57	709	23.37	1522	23.90	+
29	AD6	272	2.09	23.30	1054	21.01	35617	19.47	+
30	AE1	-	-	-	-	-	1374	25.44	-
31	AE2	-	-	34.48	21	31.33	10	46.45	-
32	AE3	-	2.19	24.26	-	37.44	4	-	+
33	AE4	-	-	-	-	37.97	4	-	-
34	AE5	1	-	-	84	26.36	3	40.56	-
35	AE6	3	-	-	7	28.35	-	-	-
36	AE7	4	-	-	1	-	1	43.34	-
37	AE8	3	-	-	1	-	2	-	-
38	AF1	28414	1.88	19.03	769	25.28	2756	25.22	+
39	AF2	3146	2.07	21.89	277	24.37	270	28.16	+
40	AF3	2046	1.97	21.49	145	25.55	254	26.67	+
41	AF4	6	-	-	56	29.20	1660	25.68	-
42	AF5	1	2.33	-	-	39.66	2	41.28	-
43	AF6	8	2.30	37.60	30	34.48	82	35.01	-
44	AG1	1	-	-	-	26.03	201	24.91	-
45	AG2	-	-	22.42	50	27.32	735	20.14	-
46	AH1	-	-	-	6	26.33	5001	20.89	-
47	AI1	-	-	-	187	26.34	111	28.37	-
48	AI2	-	-	-	177	26.11	1182	23.82	-
49	AI3	-	-	37.71	26	28.89	3791	24.29	-
50	AI4	-	-	-	138	23.78	3041	22.67	-

No	Farm	Reads *Mb*	ID *Mb*	Cq *Mb*	Reads *Mbr*	Cq *Mbr*	Reads *Md*	Cq *Md*	SIA *Mb*
51	AI5	-	-	-	18	32.99	2324	26.82	-
52	AI6	-	-	33.96	1	30.31	482	26.51	-
53	AI7	-	-	-	23	31.11	4319	24.73	-
54	AI8	12	-	22.00	37	24.05	569	23.48	+
55	AI9	-	-	-	74	24.30	207	26.04	-
56	AI10	-	-	-	88	24.10	167	26.73	-
57	AI11	1	-	-	-	36.65	9	33.96	-
58	AI12	1	-	-	42	27.31	2551	24.35	-
59	AI13	-	-	-	-	-	132	30.29	-
60	AJ1	2	-	-	74	-	863	33.27	-
61	AJ2	-	-	-	-	24.91	297	23.57	-
62	AK1	92	2.17	25.30	-	32.01	50	31.93	+
63	AK2	7312	2.20	22.80	70	31.27	67	39.18	+
64	AK3	2038	2.20	19.08	-	31.91	2467	20.94	+
65	AK4	260	2.34	28.54	111	29.17	19	35.42	+
66	AK5	7277	1.97	20.12	488	23.17	5035	22.00	+
67	AK6	21	2.30	29.83	-	35.44	28	31.73	+
68	AK7	3	2.23	28.72	-	34.35	93	27.56	+
69	AK8	186	1.91	27.12	-	37.46	281	27.32	+
70	AK9	103	2.28	23.43	1	30.28	18	27.53	+
71	AK10	5830	1.72	19.52	-	29.11	996	25.17	+
72	AK11	2102	2.18	21.63	-	34.90	55	35.53	+
73	AK12	677	1.96	21.85	2	31.04	1488	26.99	+
74	AK13	5	2.01	27.32	-	30.88	9	29.35	+
75	AK14	3	-	28.48	-	-	10	26.04	+
76	AK15	-	2.34	39.60	10	28.43	5	30.25	-
77	AK16	1467	1.79	20.63	-	38.38	546	24.25	+
78	AK17	14	2.24	25.38	-	34.67	14	30.87	+
79	AK18	-	1.95	37.39	-	37.27	6	30.61	-
80	AK19	-	2.10	30.39	-	38.57	1	31.75	+
81	AK20	-	1.95	37.41	-	35.04	1	32.71	+
82	AL1	-	2.22	33.58	18	31.36	37	31.24	+
83	AL2	-	2.38	33.00	20	32.38	1068	26.96	-
84	AL3	-	-	-	-	28.20	24	28.08	-
85	AL4	3	2.27	30.36	99	27.79	10	31.83	+
86	AL5	-	-	-	32	25.25	1115	24.07	-
87	AL6	-	-	-	257	21.09	905	22.63	-
88	AL7	-	-	38.70	534	23.25	6964	21.90	+
89	AM1	-	-	37.81	-	-	1461	27.66	-
90	AM2	-	-	-	-	-	232	31.62	-
91	AM3	-	-	-	-	-	857	22.79	-
92	AM4	-	-	-	-	-	34	32.07	-
93	AM5	-	-	-	5	28.57	83	28.52	-
94	AM6	-	-	-	-	-	1629	21.32	-
95	AN1	2	-	-	733	23.46	2633	23.18	-
96	AN2	-	-	-	-	39.68	14	29.33	-
97	AN3	-	-	-	-	-	107	27.04	-
98	AN4	-	-	-	15	24.21	262	22.75	-
99	AN5	-	-	34.09	4	33.20	1	35.00	-
100	AN6	-	-	41.15	-	30.26	174	23.98	-

Interpretation as follows: positive result when ≥ 5 reads, ID ≥ 1.7, Cq < 35 or when “oil-like” film surrounding colony is observed

Supplementary Table 2. Overview of the identification of *Mycoplasma bovis* in individual BALf (n=85) and pooled samples (n=17) by nanopore sequencing and the RIMM method.

No.	**Farm**	**Number of samples positive for *M. bovis***			
		Nanopore sequencing		RIMM	
		Individual	Pooled	Individual	Pooled
1	AB	0/5	Negative	0/5	Negative
2	AB	0/5	Negative	0/5	Negative
3	AB	0/5	Negative	0/5	Negative
4	AB/AC	0/5	Negative	0/5	Negative
5	AD	3/5	Positive	4/5	Positive
6	AE	0/5	Positive	2/5	Positive
7	AF	4/5	Positive	4/5	Positive
8	AI	0/5	Negative	0/5	Negative
9	AI	1/5	Positive	0/5	Positive
10	AI	0/5	Negative	0/5	Negative
11	AK	5/5	Positive	5/5	Positive
12	AK	4/5	Positive	5/5	Positive
13	AK	3/5	Positive	4/5	Positive
14	AK	2/5	Negative	5/5	Positive
15	AL	0/5	Negative	2/5	Positive
16	AM	0/5	Negative	0/5	Negative
17	AN	0/5	Negative	0/5	Negative

Pooled samples were categorized as positive, when at least one out of the five individual sample was positive

REFERENCES

Becker, C.A.M., Ambroset, C., Huleux, A., Vialatte, A., Colin, A., Tricot, A., Arcangioli, M.A., Tardy, F. 2020. Monitoring *Mycoplasma bovis* diversity and antimicrobial susceptibility in calf feedlots undergoing a respiratory disease outbreak. *Pathogens 9*, 593.

Bokma, J., Pardon, B., Van Driessche, L., Gille, L., Deprez, P., Haesebrouck, F., Boyen, F. 2019. Optimizing identification of *Mycoplasma bovis* by MALDI-TOF MS. *Res Vet Sci 125*, 185-188.

Bokma, J., Van Driessche, L., Deprez, P., Haesebrouck, F., Vahl, M., Weesendorp, E., Deurenberg, R.H., Pardon, B., Boyen, F. 2020a. Rapid identification of *Mycoplasma bovis* from bovine bronchoalveolar lavage fluid with MALDI-TOF MS after enrichment procedure. *J Clin Microbiol 58*, e00004-20.

Bokma, J., Vereecke, N., De Bleecker, K., Callens, J., Ribbens, S., Nauwynck, H., Haesebrouck, F., Theuns, S., Boyen, F., Pardon, B. 2020b. Phylogenomic analysis of *Mycoplasma bovis* from Belgian veal, dairy and beef herds. *Vet Res 51*, 121.

Branscum, A.J., Gardner, I.A., Johnson, W.O. 2005. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. *Prev Vet Med 68*, 145-63.

Broccolo, F., Scarpellini, P., Locatelli, G., Zingale, A., Brambilla, A.M., Cichero, P., Sechi, L.A., Lazzarin, A., Lusso, P., Malnati, M.S. 2003. Rapid diagnosis of mycobacterial infections and quantitation of *Mycobacterium tuberculosis* load by two real-time calibrated PCR assays. *J Clin Microbiol 41*, 4565-4572.

Buskirk, A.D., Hettick, J.M., Chipinda, I., Law, B.F., Siegel, P.D., Slaven, J.E., Green, B.J., Beezhold, D.H. 2011. Fungal pigments inhibit the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of darkly pigmented fungi. *Anal Biochem 411*, 122-8.

Caswell, J.L., Bateman, K.G., Cai, H.Y., Castillo-Alcala, F. 2010. *Mycoplasma bovis* in respiratory disease of feedlot cattle. *Vet Clin North Am Food Anim Pract 26,* 365-379.

Cornelissen, J.B.W.J., de Bree, F.M., van der Wal, F.J., Kooi, E.A., Koene, M.G.J., Bossers, A., Smid, B., Antonis, A.F., Wisselink, H.J. 2017. *Mycoplasma* detection by triplex real-time PCR in bronchoalveolar lavage fluid from bovine respiratory disease complex cases. *BMC Vet Res 13,* 97

Dudek, K., Bednarek, D., Ayling, R.D., Kycko, A., Szacawa, E., Karpińska, T.A. 2016. An experimental vaccine composed of two adjuvants gives protection against *Mycoplasma bovis* in calves. *Vaccine 34,* 3051-3058.

Ferreira, L., Sánchez-Juanes, F., Muñoz-Bellido, J.L., González-Buitrago, J.M. 2011. Rapid method for direct identification of bacteria in urine and blood culture samples by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: Intact cell vs. extraction method. *Clin Microbiol Infect 17*, 1007-12.

Gautier-Bouchardon, A. V. 2018. Antimicrobial Resistance in *Mycoplasma* spp. *Microbiol Spectr 6*.

Genova, S.G., Streeter, R.N., Velguth, K.E., Snider, T.A., Kocan, K.M., Simpson, K.M. 2011. Severe anemia associated with *Mycoplasma wenyonii* infection in a mature cow. *Can Vet J 52*, 1018-1021.

Gille, L., Callens, J., Supré, K., Boyen, F., Haesebrouck, F., Van Driessche, L., van Leenen, K., Deprez, P., Pardon, B. 2018. Use of a breeding bull and absence of a calving pen as risk factors for the presence of *Mycoplasma bovis* in dairy herds. *J Dairy Sci 101*, 8284-8290.

Grau, O., Slizewicz, B., Tuppin, P., Launay, V., Bourgeois, E., Sagot, N., Moynier, M., Lafeuillade, A., Bachelez, H., Clauvel, J.P., Blanchard, A., Bahraoui, E., Montagnier, L. 1995. Association of *Mycoplasma penetrans* with human immunodeficiency virus infection. *J Infect Dis 172*, 672-681.

Hoelzle, K., Ade, J., Hoelzle, L.E. 2020. Persistence in Livestock Mycoplasmas—a Key Role in Infection and Pathogenesis. *Curr Clin Microbiol Reports 7*, 81-89.

Kinnear, A., McAllister, T.A., Zaheer, R., Waldner, M., Ruzzini, A.C., Andrés-Lasheras, S., Parker, S., Hill, J.E., Jelinski, M.D. 2020. Investigation of macrolide resistance genotypes in *Mycoplasma bovis* isolates from canadian feedlot cattle. *Pathogens 9*, 622.

Ledger, L., Eidt, J., Cai, H.Y. 2020. Identification of antimicrobial resistance-associated genes through whole genome sequencing of *Mycoplasma bovis* isolates with different antimicrobial resistances. *Pathogens 9*, 588.

Lohse, S., Pfuhl, T., Berkó-Göttel, B., Rissland, J., Geißler, T., Gärtner, B., Becker, S.L., Schneitler, S., Smola, S. 2020. Pooling of samples for testing for SARS-CoV-2 in asymptomatic people. *Lancet Infect Dis 20*, 1231-1232.

Lysnyansky, I., Ayling, R.D. 2016. *Mycoplasma bovi*s: Mechanisms of resistance and trends in antimicrobial susceptibility. *Front Microbiol 27*, 595.

Maunsell, F.P., Donovan, G.A. 2009. *Mycoplasma bovis* infections in young calves. *Vet Clin North Am Food Anim Pract 25*, 139-177.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle. *J Vet Intern Med 25*, 772-783.

Maurin, M. 2012. Real-time PCR as a diagnostic tool for bacterial diseases. *Expert Rev Mol Diagn 12*, 731-754.

McAuliffe, L., Ellis, R.J., Miles, K., Ayling, R.D., Nicholas, R.A.J. 2006. Biofilm formation by *Mycoplasma* species and its role in environmental persistence and survival. *Microbiology 152,* 913-922.

McCabe, M.S., Cormican, P., Johnston, D., Earley, B. 2018. Simultaneous detection of DNA and RNA virus species involved in bovine respiratory disease by PCR-free rapid tagmentation-based library preparation and MinION nanopore sequencing. *bioRxiv*.

Murai, K., Lehenbauer, T.W., Champagne, J.D., Glenn, K., Aly, S.S. 2014. Cost-effectiveness of

diagnostic strategies using quantitative real-time PCR and bacterial culture to identify contagious mastitis cases in large dairy herds. *Prev Vet Med 113*, 522-535.

Nicholas, R.A.J., Ayling, R.D., Stipkovits, L.P. 2002. An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis*: clinical, cultural, serological and pathological findings. *Vaccine 20*, 3569-3575.

Nilsson, A.C., Björkman, P., Welinder-Olsson, C., Widell, A., Persson, K. 2010. Clinical severity of *Mycoplasma pneumoniae* (MP) infection is associated with bacterial load in oropharyngeal secretions but not with MP genotype. *BMC Infect Dis 10*, 39.

Nouvel, L.X., Hygonenq, M.C., Catays, G., Martinelli, E., Le Page, P., Collin, É., Inokuma, H., Schelcher, F., Citti, C., Maillard, R., 2019. First detection of *Mycoplasma wenyonii* in France: Identification, evaluation of the clinical impact and development of a new specific detection assay. *Comp. Immunol. Microbiol. Infect. Dis 63*, 148-153.

Oliveira, T.E.S., Pelaquim, I.F., Flores, E.F., Massi, R.P., Valdiviezo, M.J.J., Pretto-Giordano, L.G., Alfieri, A.A., Saut, J.P.E., Headley, S.A. 2020. *Mycoplasma bovis* and viral agents associated with the development of bovine respiratory disease in adult dairy cows. *Transbound Emerg Dis 67*, 82-93.

Pardon, B., Buczinski, S. 2020. Bovine respiratory disease diagnosis: what progress has been made in infectious diagnosis? *Vet Clin North Am Food Anim Pract 36*, 425-444.

Pardon, B., Callens, J., Maris, J., Allais, L., Van Praet, W., Deprez, P., Ribbens, S. 2020. Pathogen-specific risk factors in acute outbreaks of respiratory disease in calves. *J Dairy Sci 103,* 2556-2566.

Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House, J.K. 2018. A review of mycoplasma diagnostics in cattle. *J Vet Intern Med 32*, 1241-1252.

Pereyre, S., Tardy, F., Renaudin, H., Cauvin, E., Del Prá Netto Machado, L., Tricot, A., Benoit, F., Treilles, M., Bébéar, C. 2013. Identification and subtyping of clinically relevant human and ruminant mycoplasmas by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol 51*, 3314-3323.

Perez-Casal, J. 2020. Pathogenesis and Virulence of *Mycoplasma bovis*. *Vet Clin Food Anim 36*, 269-278.

Preiswerk, B., Imkamp, F., Vorburger, D., Hömke, R. V., Keller, P.M., Wagner, K. 2020. *Mycoplasma penetrans* bacteremia in an immunocompromised patient detected by metagenomic sequencing: A case report. *BMC Infect Dis 20*, 7.

Randall, L.P., Lemma, F., Koylass, M., Rogers, J., Ayling, R.D., Worth, D., Klita, M., Steventon, A., Line, K., Wragg, P., Muchowski, J., Kostrzewa, M., Whatmore, A.M. 2015. Evaluation of MALDI-ToF as a method for the identification of bacteria in the veterinary diagnostic laboratory. *Res Vet Sci 101*, 42-49.

Razin, S., Hayflick, L. 2010. Highlights of mycoplasma research - An historical perspective.

Biologicals 38, 183-190.

Rijckaert, J., Raes, E., Buczinski, S., Dumoulin, M., Deprez, P., Van Ham, L., van Loon, G., Pardon, B. 2020. Accuracy of transcranial magnetic stimulation and a Bayesian latent class model for diagnosis of spinal cord dysfunction in horses. *J Vet Intern Med 34*, 964-971.

Smith, J.A., Thrall, M.A., Smith, J.L., Salman, M.D., Ching, S. V., Collins, J.K., 1990. *Eperythrozoon wenyonii* infection in dairy cattle. *J Am Vet Med Assoc 196*, 1244-1250.

Spergser, J., Hess, C., Loncaric, I., Ramírez, A.S. 2019. Matrix-assisted laser desorption ionization-time of flight mass spectrometry is a superior diagnostic tool for the identification and differentiation of mycoplasmas isolated from animals. *J Clin Microbiol 57,* e00316-19

Tagawa, M., Matsumoto, K., Inokuma, H. 2008. Molecular detection of *Mycoplasma wenyonii* and "*Candidatus Mycoplasma haemobos*" in cattle in Hokkaido, Japan. *Vet Microbiol 132*, 177-180.

Takahashi, T., Tamura, M., Takahashi, S.N., Matsumoto, K., Sawada, S., Yokoyama, E., Nakayama, T., Mizutani, T., Takasu, T., Nagase, H. 2007. Quantitative nested real-time PCR assay for assessing the clinical course of tuberculous meningitis. *J Neurol Sci 255*, 69-76.

Theuns, S., Vanmechelen, B., Bernaert, Q., Deboutte, W., Vandenhole, M., Beller, L., Matthijnssens, J., Maes, P., Nauwynck, H.J. 2018. Nanopore sequencing as a revolutionary diagnostic tool for porcine viral enteric disease complexes identifies porcine kobuvirus as an important enteric virus. *Sci Rep 8*, 9830.

Thomas, A., Ball, H., Dizier, I., Trolin, A., Bell, C., Mainil, J., Linden, A. 2002. Isolation of *Mycoplasma* species from the lower respiratory tract of healthy cattle and cattle with respiratory disease in Belgium. *Vet Rec 151*, 472-476.

Timsit, E., Workentine, M., van der Meer, F., Alexander, T. 2018. Distinct bacterial metacommunities inhabit the upper and lower respiratory tracts of healthy feedlot cattle and those diagnosed with bronchopneumonia. *Vet Microbiol 221*, 105-113.

Van Driessche, L., Valgaeren, B.R., Gille, L., Boyen, F., Ducatelle, R., Haesebrouck, F., Deprez, P., Pardon, B. 2017. A deep nasopharyngeal swab versus nonendoscopic bronchoalveolar lavage for isolation of bacterial pathogens from preweaned calves with respiratory disease. *J Vet Intern Med 31*, 946-953.

Wisselink, H.J., Smid, B., Plater, J., Ridley, A., Andersson, A.M., Aspán, A., Pohjanvirta, T., Vähänikkilä, N., Larsen, H., Høgberg, J., Colin, A., Tardy, F. 2019. A European interlaboratory trial to evaluate the performance of different PCR methods for *Mycoplasma bovis* diagnosis. *BMC Vet Res 15*, 86.

Yair, Y., Borovok, I., Mikula, I., Falk, R., Fox, L.K., Gophna, U., Lysnyansky, I. 2020. Genomics-based epidemiology of bovine *Mycoplasma bovis* strains in Israel. *BMC Genomics 21*, 70.

Yáñez, A., Cedillo, L., Neyrolles, O., Alonso, E., Prévost, M.C., Rojas, J., Watson, H.L., Blanchard, A., Cassell, G.H. 1999. *Mycoplasma penetrans* bacteremia and primary antiphospholipid syndrome. *Emerg Infect Dis 5*, 164-167.

CHAPTER 4

PHYLOGENOMIC ANALYSIS OF MYCOPLASMA BOVIS FROM BELGIAN VEAL, DAIRY, AND BEEF HERDS

PHYLOGENOMIC ANALYSIS OF *MYCOPLASMA BOVIS* FROM BELGIAN VEAL, DAIRY AND BEEF HERDS

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ABSTRACT

M. bovis is one of the leading causes of respiratory disease and antimicrobial use in cattle. The pathogen is widespread in different cattle industries worldwide, but highest prevalence is found in the veal industry. Knowledge on *M. bovis* strain distribution over the dairy, beef and veal industries is crucial for the design of effective control and prevention programs, but currently undocumented. Therefore, the present study evaluated the molecular epidemiology and genetic relatedness of *M. bovis* isolates obtained from Belgian beef, dairy and veal farms, and how these relate to *M. bovis* strains obtained worldwide. Full genomes of one hundred Belgian *M. bovis* isolates collected over a 5-year period (2014-2019), obtained from 27 dairy, 38 beef and 29 veal farms, were sequenced by long-read nanopore sequencing. Consensus sequences were used to generate a phylogenetic tree in order to associate genetic clusters with cattle sector, geographical area and year of isolation. The phylogenetic analysis of the Belgian *M. bovis* isolates resulted in 5 major clusters and 1 outlier. No sector-specific *M. bovis* clustering was identified. On a world scale, Belgian isolates clustered with Israeli, European and American strains. Different *M. bovis* clusters circulated for at least 1.5 consecutive years throughout the country, affecting all observed industries. Therefore, the high prevalence in the veal industry is more likely the consequence of frequent purchase from the dairy and beef industry, than that a reservoir of veal specific strains on farm would exist. These results emphasize the importance of biosecurity in *M. bovis* control and prevention.

Keywords: cattle, long-read nanopore sequencing, phylogenetic analysis, SNP analysis, whole genome

INTRODUCTION

Mycoplasma bovis (*M. bovis*) causes mostly pneumonia, arthritis, otitis in calves and mastitis in adult cattle (Maunsell and Donovan, 2009; Maunsell et al., 2011) resulting in high antimicrobial use (AMU) and enormous economic losses in cattle farming sectors worldwide (Nicholas and Ayling, 2003; Fox et al., 2005; Maunsell and Donovan, 2009). In Belgium, 100% of the veal farms are seropositive for *M. bovis* (Pardon et al., 2011; Pardon, 2012), whereas *M. bovis* is involved in 33% of acute pneumonia outbreaks in beef and dairy farms (Pardon et al., 2020). Treatment of *M. bovis* is frequently unsatisfactory, probably due to a combination of intrinsic and acquired antimicrobial resistance, immuno-evasive properties of the pathogen and failure of the animal to generate an effective immune response (Gautier-Bouchardon, 2018; Maunsell and Chase, 2019). Together with the absence of an effective commercially available vaccine, the control of *M. bovis* is particularly challenging.

A contemporary fear is that the veal sector, currently combining a high AMU and a farm level *M. bovis* prevalence of 100%, is a reservoir for multi-resistant sector-specific *M. bovis* strains (Arcangioli et al., 2008; Pardon, 2012; Bokma et al., 2019). Currently, there is insufficient knowledge about the epidemiology of circulating *M. bovis* strains to answer this question. Several epidemiological studies observed clonal emergence and identified dominant lineages of the *M. bovis* bacterium, based on antimicrobial resistance patterns and different strain typing methods (Gautier-Bouchardon et al., 2014; Becker et al., 2015; Rosales et al., 2015; Bürki et al., 2016). In the past, different approaches were used to subtype *M. bovis* strains, including random amplification of polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), amplification fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), multiple-locus variable-number tandem repeat (MLVA), and multi-locus sequence typing (MLST) (Butler et al., 2001; McAuliffe et al., 2004; Spergser et al., 2013; Becker et al., 2015; Rosales et al., 2015). Unfortunately, results from these typing methods are difficult to compare and only focus on a small fraction of the genomic information, resulting in a limited insight in the genetics and incongruence among studies (Maiden et al., 1998; McAuliffe et al., 2014; Register et al., 2020). Therefore, whole genome sequencing (WGS) could be a great opportunity, considering its highly discriminative capacity and reproducibility compared to older typing methods (Leekitcharoenphon et al., 2014; Deng et al., 2016).

Several studies already investigated whether specific *M. bovis* strains were associated with affected organs, such as udder, respiratory tract or joints (Register et al., 2015; Rosales et al., 2015; Parker et al., 2016), geographical location (Register et al., 2015; Parker et al., 2016) or health status (Register et al., 2015; Yair et al., 2020). Only one study determined epidemiology based on AP-PCR in three farms from three different husbandry conditions (dairy calf ranch, feedlot and closed beef herd), presuming that management factors could influence the distribution of *M. bovis* (Butler et al., 2001). These husbandry conditions are not comparable with the three main sectors in Europe. In Europe, a lot of short-distance movements of cattle between farms is seen, and the veal industry is an important side market of the dairy and beef industry (Ensoy et al., 2014; Pardon et al., 2014). It is currently not clear whether sector-specific *M. bovis* strains are present and what their genetic relation is to previously sequenced *M. bovis* isolates. Therefore, the present study first evaluated the molecular epidemiology and genetic relatedness of *M. bovis* isolates obtained from Belgian beef, dairy and veal farms. Furthermore, it studied the relationship of these isolates to *M. bovis* strains from other countries.

MATERIALS AND METHODS

Mycoplasma bovis collection and identification

One hundred *M. bovis* isolates were obtained from 94 Belgian farms (27 dairy, 38 beef and 29 veal) over a 5-year period (2014-2019). All isolates were obtained from diagnostic samples collected by field veterinarians from clinical cases, in compliance with EU legislation on ethics in animal experimentation [2010/63/EU]. Isolates were collected in 2014 (n = 1), 2016 (11), 2017 (63), 2018 (19) and 2019 (6), originating from the provinces East-Flanders (n = 10), West-Flanders (25), Antwerp (38), Limburg (6), Flemish Brabant (10), Heynowes (6), Namen (2) and Liege (1). The origin of two isolates was unclear. The samples were retrieved from the respiratory tract (89), middle ear (3), milk (4), joint (2) and other fluids (3) of calves and adult cattle, as shown in Supplement 1. The samples were cultured on selective indicative agar (Bokma et al., 2020), and identification was verified with MALDI-TOF MS (score value ≥ 1.7), as described earlier (Bokma et al., 2019) and Kraken2 analysis (v2.0.8; Wood et al., 2019). Isolates had a passage history of maximum 3-5 times and all isolates were stored at maximum -20°C until further analysis.

Preparation and DNA extraction

In ten separate runs, all *M. bovis* isolates were thawed and cultured in 10 mL modified PPLO broth (pH 7.8) (Difco™, BD Diagnostic Systems, Sparks, Md.), supplemented with 25% inactivated horse serum (Gibco™), 0.7% technical yeast extract, 0.5% sodium pyruvate (ReagentPlus, Sigma-Aldrich®), 0.5% D-(+)-glucose monohydrate (Sigma-Aldrich) and 0.005% phenol red. After 4 days of incubation (37ºC, 5% CO_2) a bacterial suspension of approximately 10^8 CFU/mL was obtained. Bacterial DNA was obtained using the ZymoBIOMICS DNA Miniprep kit (Zymo Research) according to the manufacturer's instructions. Quantity and quality were verified using NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Low quality samples were further cleaned using CleanNGS (CleanNa) beads. All runs included the *M. bovis* PG45 type strain (ATCC 25523) and modified PPLO broth as the positive and negative control, respectively.

Library preparation and MinION long-read sequencing

Quality-checked native *M. bovis* DNA was immediately used for library preparation using the Rapid Barcoding Sequencing Kit (SQK-RBK004; Oxford Nanopore Technologies (ONT)), following manufacturers' instructions. For each run, ten field strains, one positive control (PG45) and one negative control (sterile broth) were multiplexed (400 ng DNA per sample). A new R9.4.1 Flow cell (ONT) was used for a 48h sequencing run on MinION device (ONT). Raw fast5 read files were collected using MinKnow v.3.6.5.

Bioinformatics pipeline

All data were analyzed on an Ubuntu 18.04.3 LTS system. In order to speed up bioinformatics analyses, GPU resources (GeForce RTX 2080 Ti/PCIe/SSE2) were exploited where possible. Raw fast5 files were basecalled using Guppy basecaller (GPU v.3.3.0; ONT), followed by demultiplexing, adapter trimming, and quality filtering (Q-score ≥ 7) of fastq files with qcat (v.1.1.0; ONT) and NanoFilt (v. 2.5.0; De Coster et al., 2018), respectively. Reference-based assemblies were generated using the *M. bovis* PG45 type strain sequence (NC_014760.1) by mapping filtered reads onto the reference using GraphMap (v.0.5.2; Sović et al., 2016). Final consensus sequences were generated using Medaka (GPU v.0.10.0; ONT). All strains were identified as *M. bovis* using Kraken2 (v2.0.8; Wood et al., 2019) by aligning the reads against the minikraken_v1_8GB database with standard settings. Overall consensus assembly accuracies were verified by comparing total Single Nucleotide Polymorphisms (SNPs) using the CSI phylogeny package (v1.4, Center for Genomic Epidemiology, Denmark; Kaas et al.,

2014) as compared to the *M. bovis* PG45 type strain (NC_014760.1) reference sequences. To validate the use of long-read sequencing, SNPs of ten independent *M. bovis* PG45 assemblies were compared to those in a single MiSeq experimental dataset. All *M. bovis* consensus genomes are available for download on the NCBI GenBank database under the BioProject PRJNA639688 and accession numbers (SAMN15246515-SAMN1524662). Sequencing summaries can be found in Supplement file 1.

Phylogenetic analysis

Phylogenetic analysis was performed on all newly generated consensus sequences alone or in combination with 250 previously published *M. bovis* sequences using the FastTree-based CSI Phylogeny v1.4 (see Supplement 2). All analyses included the *M. bovis* PG45 type strain (NC_014760.1) as reference and *M. agalactiae* PG2 (NC_009497.1) as outgroup. Resulting Newick files were visualized with MEGA-X software (Kumar et al., 2018).

Cluster and strain determination

Due to the lack of relatedness criteria for SNP typing schemes of *M. bovis* and the need to establish these per organism and experimental design (Schürch et al., 2018), clusters were defined by visual inspection of the phylogenetic tree and by taking into account bootstrap support. In addition, the matrix of pairwise SNP counts was extracted from CSI Phylogeny for further inspection. Mean SNP differences were calculated between within-cluster isolates, and outliers were defined with the 1.5xIQR rule, using the Outlier Calculator (https://miniwebtool.com/outlier-calculator/).

Geographical distribution

Esri®ArcMap™ (version 10.7.1) software was used to visualize the geographical distribution and density of *M. bovis* isolates over Belgium. Herd size was based on the national Identification and Registration system, containing, on the first of January 2017, a total of 23,995 cattle herds in Belgium (23,733 conventional herds; 262 veal), and a total of 2,517,850 cattle. The spatial distribution of the Belgian cattle (both cattle and veal calves) was displayed using kernel smoothing. Coordinates of Belgian cattle herds were converted into a continuous raster using the kernel density estimation function weighted by number of cattle (Spatial Analyst, ArcMAP X, ESRI, Redlands, CA, USA).

RESULTS

Phylogenetic analysis of Belgian isolates

A median sequencing depth of 618X (range: 32X-2689X) was obtained from long reads with an average N_{50} read length of 5,706 ± 1,514 bp for all Belgian *M. bovis* isolates. First, implementation of long-read sequencing of *M. bovis* genomes was validated by comparing total SNPs from ten independently sequenced *M. bovis* PG45 sequences and a single *M. bovis* PG45 MiSeq dataset, showing 53 ± 3 SNPs and 27 SNPs difference, respectively, compared with the 1,003,404 bp of the *M. bovis* PG45 reference genome (NC_014760.1). The observed average SNP difference of 0.005% for the long-read sequencing approach was considered acceptable to allow meaningful phylogenetic analyses. In addition, control strain *M. bovis* PG45 results were mutually compared over all ten runs, showing a mean SNP difference of 20 (range 8-30, standard deviation 4.6), which was also demonstrated an acceptable inter-experimental variation. Taking into account all Belgian *M. bovis* strains, and also including the outgroup *M. agalactiae,* 51.4% of the *M. bovis* genome or 515,324 nucleotide positions were used for phylogeny. The minimum and maximum SNP differences among Belgian *M. bovis* isolates were 33 and 3,775, respectively.

Table 1. Pairwise SNP differences between *M. bovis* isolates within Belgian cluster I to V and VK30.

Cluster	*Min SNP*	*Max SNP*	*ΔSNP*	*Mean*	*SD*
I	292	1126	834	843	427
VK30[a]	3221	3775	3436	3457	95
II	78	165	87	135	22
III	60	376	316	198	71
IV	76	1103	1027	245	251
V	77	1512	1435	445	267

Δ: difference between minimum (min) and maximum (max) pairwise SNPs; SD: standard deviation; [a] VK30 was compared to cluster I to V.

Visual inspection of the phylogenetic analysis of 100 *M. bovis* isolates resulted in 5 clusters (I-V): 3 large clusters (n ≥ 10 isolates), 2 smaller clusters (n < 10), and 1 distinct strain (VK30) as shown in Figure 1. Cluster I to V contained 3, 7, 16, 33, and 40 *M. bovis* isolates, respectively. Inspection of pairwise SNP differences per cluster, showed more homogeny within clusters II and III (mean ΔSNPs of 87 and 316, respectively), compared with cluster I, IV and V (mean ΔSNPs of 834, 1027, and 1435) (Table 1). Mean SNP differences among within-cluster isolates and outlier calculations showed outliers within cluster III (Mb222, Mb231), IV (Mb201, Mb240, Mb175, TOVK) and V (Mb116, Mb166, VK11, VK23).

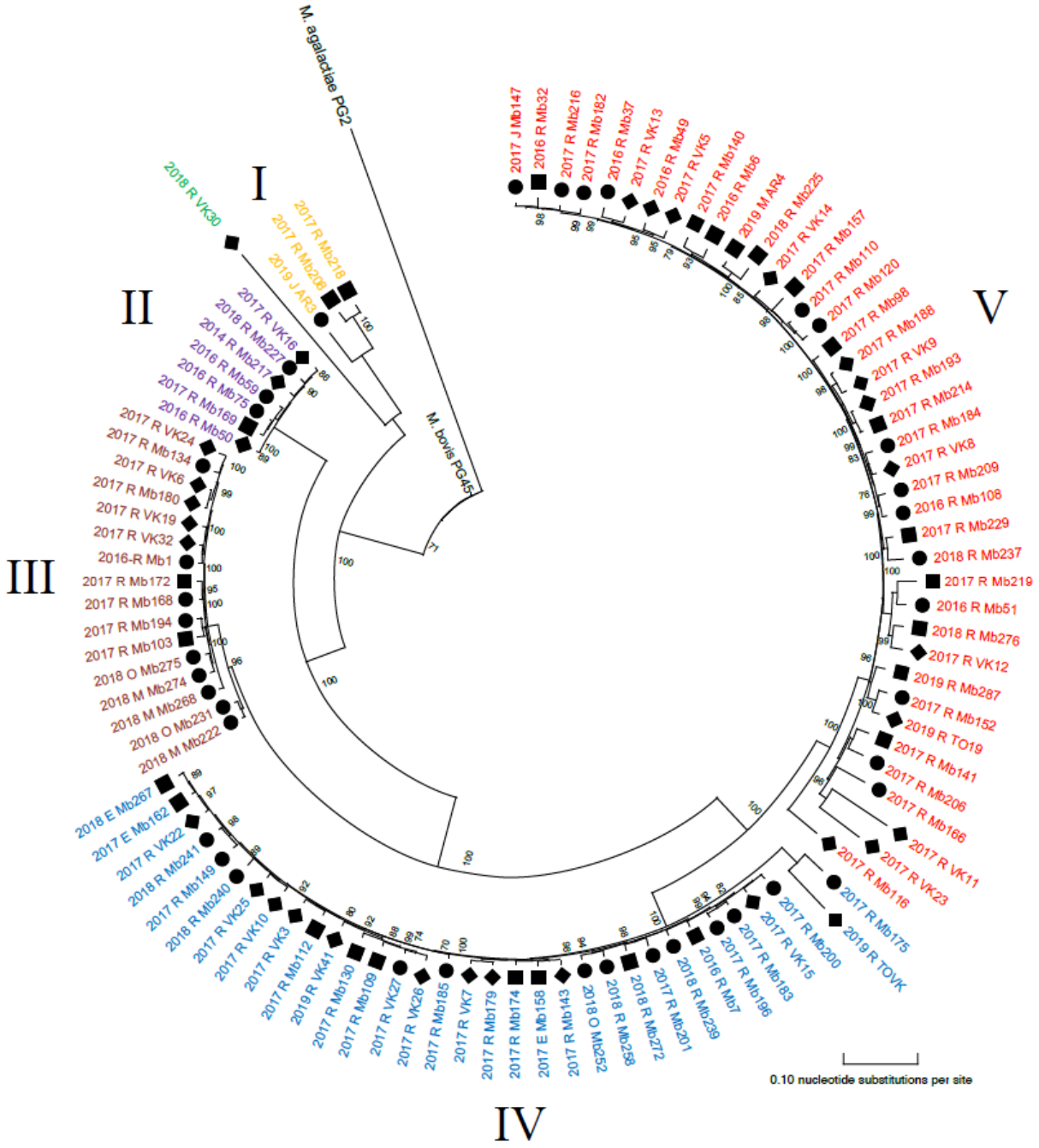

Figure 1. SNP-based phylogenetic tree of 100 *M. bovis* isolates from Belgian dairy, beef and veal farms. The figure was created using MEGA-X software with *M. bovis* isolates obtained over 2014-2019. The tree was rerooted to *M. agalactiae* PG2, which was included as an outgroup. Clusters (I-V), and VK30 are represented by different colors. The designation of the isolates features the sector (■ dairy; ● beef; ♦ veal), year of isolation (2014-2019), affected organ (R: respiratory tract; M: milk; E: ear, J: joint and O: other) and sequence identification (see Supplement file 1). The scale bar indicates the number of substitutions per site, and bootstrap values are represented on nodes.

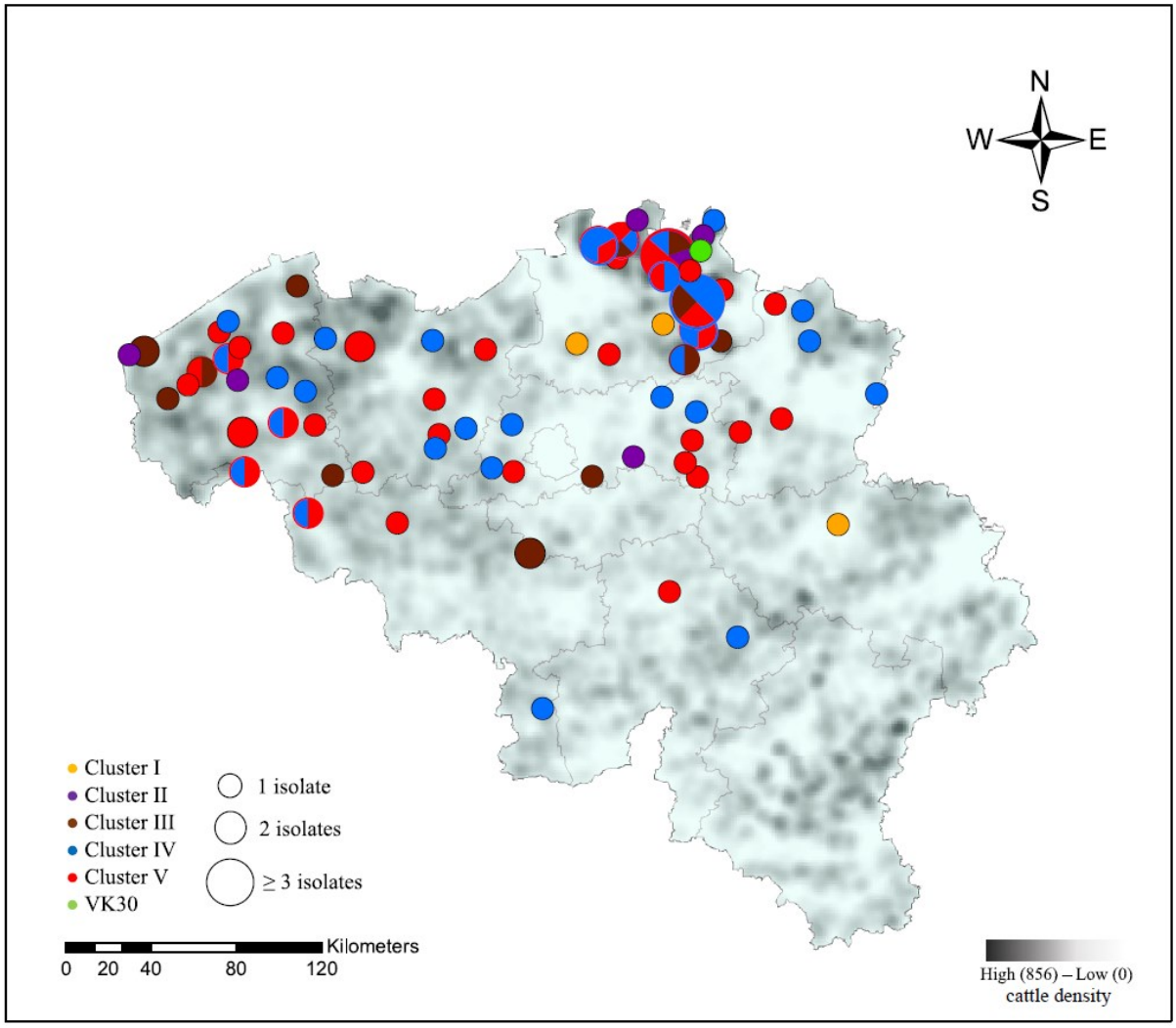

Figure 2. Geographical distribution of different *M. bovis* clusters over 2014-2019 and cattle density in Belgium (2017). The map was created using Esri®ArcMap™ (version 10.7.1) software. Clusters (I-V) are represented by different colors and the radius of the circle represents the number of isolates from one village. Mixed colors within one circle represent the presence of different clusters within one village.

Between and within clusters, no association could be observed for the different cattle sectors or year of isolation (Fig. 1). Two different isolates from the same herd (veal) and same sampling period (Mb49 and Mb50) did not cluster together (II and V). All clusters persisted in Belgium for at least 1.5 consecutive years throughout the country. *M. bovis* strains isolated from the middle ear (n = 3) were clustered within cluster IV, while those obtained from milk, joint and other samples were scattered over different clusters (Fig. 1). Finally, no clear association between geographic location of sampled farms and *M. bovis* clusters was observed, as shown in Figure 2.

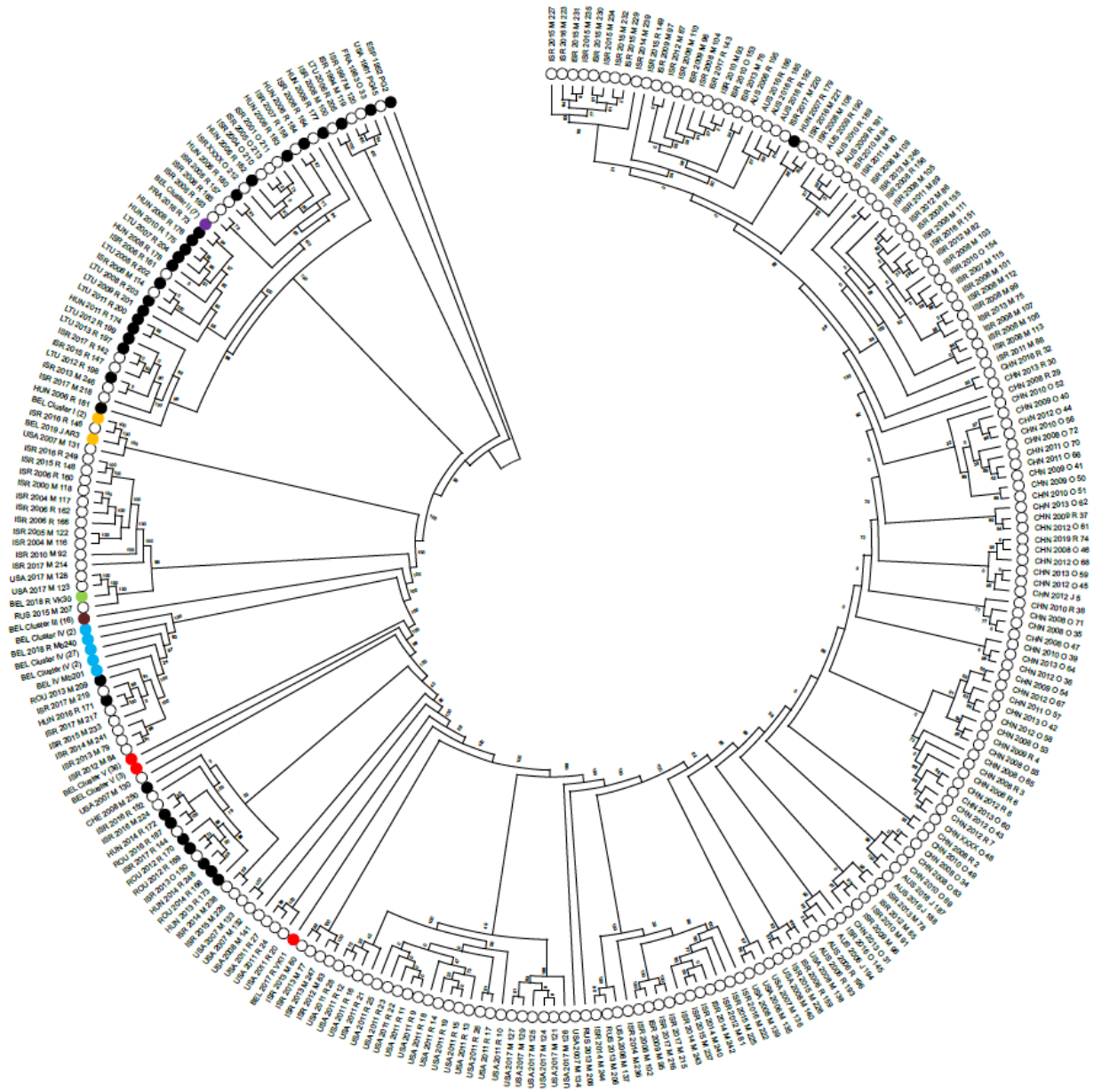

Figure 3. SNP-based topology of 350 *M. bovis* isolates in MEGA-X. The tree was rerooted to *M. agalactiae* PG2 (EPS 1952 PG2), which was included as outgroup. Belgian clusters (I-V) were collapsed as far as possible and represented by different colors (I: yellow; II: purple; III: brown; IV: blue, V: red; VK30: green). The designation of the isolates contains the coded name of country of origin (ISO 3166-1; Alpha-3 code), continent of origin (● Europe; o Non-Europe), year of isolation (1952-2019), affected organ (R: respiratory tract; M: milk; E: ear, J: joint and O: other or unknown) and sequence identification (see Supplement 2) or the number of collapsed Belgian isolates between brackets. Bootstrap values are represented on nodes. (full version: https://doi.org/10.1186/s13567-020-00848-z)

Phylogenetic analysis of *M. bovis* worldwide

All 100 Belgian isolates were added to the worldwide phylogenetic tree. The percentage of the reference genome covered by all isolates, including the PG45 standard strain and the *M. agalactiae* outgroup strain PG2 was 39.3%, therefore 394,303 positions were found in all analyzed genomes. The minimum and maximum SNP differences among all *M. bovis* isolates including the reference strain, were 0 and 4,871, respectively. Belgian clusters are situated in different parts of the phylogenetic tree worldwide (Fig. 3). Cluster I is related to strains isolated in the USA (2007; mean ΔSNPs of 636) and Israel (2016; mean ΔSNPs of 1369). Cluster II is closely related to one recent French strain (2016; mean ΔSNPs of 171) and is situated in a larger cluster related to older strains from Israel and Eastern Europe (2001-2009; ΔSNPs < 200), and other more recently isolated strains from Israel and Eastern Europe (2011-2017; ΔSNPs < 500). Belgian cluster III and V do not cluster together with non-Belgian isolates, while cluster IV is closely related (ΔSNPs < 300 without cluster IV outliers) to *M. bovis* strains obtained in Israel (2012-2017) and Eastern Europe (2013-2016). VK11 remains an outlier that does not collate with the rest of cluster V. Consistent with Figure 1, VK30 is well separated from the other Belgian isolates and is very closely related (mean ΔSNPs of 171) to strains obtained from milk in the USA (2017).

DISCUSSION

In this study, one-hundred *M. bovis* isolates from different Belgian cattle sectors (beef, dairy or veal) were phylogenetically compared to investigate whether sector-specific strains exist and whether such strains are related to *M. bovis* strains previously isolated and sequenced worldwide.

In this study, we chose to apply the ONT long-read sequencing approach (Goodwin et al., 2016), because no default WGS approaches are defined for *Mycoplasma* spp. and short-read sequencing biases have been described for genomes with highly repetitive regions.

WGS approaches have become more attractive over the last years, as the cost for next-generation sequencing has significantly reduced. Single Nucleotide Polymorphism (SNP) analysis using Illumina short read data from *M. bovis* isolates already showed to be an effective way for *M. bovis* genotyping (Parker et al., 2016; Yair et al., 2020). Long-read nanopore sequencing (Oxford Nanopore Technologies) is known to create much faster results, and was recently applied in veterinary medicine as well (Quick et al., 2015; Theuns et al., 2018). Yet, lower single read accuracies are currently obtained with ONT in comparison to

Illumina (Rang et al., 2018). Therefore, the implementation of long-read sequencing to generate *M. bovis* genome assemblies was verified, showing only on average 53 SNPs difference of the long-read approach with the publically available *M. bovis* PG45 genome, representing an acceptable error rate of 0.005%. As a result, the authors believe that nanopore sequencing is a highly accessible tool, allowing practical use outside academia in routine diagnostics and real time surveillance.

From the study results, several interesting observations were made. First of all, the obtained *M. bovis* isolates belonged to at least five different *M. bovis* clusters, of which three dominant clusters were identified. This is in agreement with an Israeli study based on WGS-SNP, where six clusters were observed of which three were dominant. Remarkably, one cluster contained more than 50% of the isolates in that study (Yair et al., 2020). Several other studies also showed one or two dominant lineages, although different typing methods were used (Spergser et al., 2013; Becker et al., 2015; Bürki et al., 2016; Parker et al., 2016; Menghwar et al., 2017). In contrast to Aebi et al. (2012), where mostly herd-specific *M. bovis* isolates were seen in Switzerland, we observed close relatedness of *M. bovis* isolates over the different herds. This might be a result of more frequent purchasing cattle from different origins and transportation in Belgium, because 40% of cattle is transported at least once (and up to eight times) over a 5-year lifespan in Belgium (McAuliffe et al., 2004; Soehnlen et al., 2011; Spergser et al., 2013; Ensoy et al., 2014; Pardon et al., 2020). As such, the higher heterogeneity observed in cluster I, IV and V compared with clusters II and III, may be caused by different rates of genetic drift between clonal lines (McAuliffe et al., 2004).

Secondly, no sector-specific strains or clusters were identified in the present study, which does not entirely come as a surprise. In Belgium, veal calves are purchased from both dairy and beef farms, and fattened and slaughtered in specialized veal farms and slaughterhouses, respectively (Pardon et al., 2014). Also, approximately 15-20% of the farms in Belgium are mixed farms. Therefore, contact among different cattle sectors is intense. In addition, herd visitors and artificial insemination might play a role in spreading of *M. bovis* or introducing new strains on farms (Gonzalez et al., 1992; Gille et al., 2016; Haapala et al., 2018).

Thirdly, when we take a closer look on how the different clusters have spread over Belgium, no clear association with location was observed. This was also concluded in studies performed in the UK and USA (McAuliffe et al., 2004; Soehnlen et al., 2011; Register et al., 2015). Nevertheless, in the provinces of Antwerp and Western Flanders seem to be hotspots for *M. bovis* outbreaks. Besides a high number of local transports, Antwerp and the Flanders area are also the main gates for cattle import, which makes these areas predisposed for the

introduction of new *M. bovis* strains (Ensoy et al., 2014). Unfortunately, *M. bovis* genomes were not available for isolates obtained from the top import countries for Belgium, which are Germany and the Netherlands.

Although this study was not designed to draw definitive conclusions about year of isolation and affected organs, some preliminary observations can be made. For example, no association between *M. bovis* strain and year of isolation was observed. On the other hand, we saw that representatives of all clusters persisted for at least 1.5 consecutive years on Belgian territory. The persistence of strains within a country or herd has been described before (Butler et al., 2001; Aebi et al., 2012; Parker et al., 2016). Furthermore, shifts between dominant lineages from older to new strains have been reported before as well (Arcangioli et al., 2012; Becker et al., 2015; Bürki et al., 2016; Hata et al., 2019). Also, we did not observe an association between cluster and affected organ, which is in line with previous studies (Register et al., 2015; Rosales et al., 2015; Parker et al., 2016). Yet, it was remarkable that all isolates obtained from the middle ear were clustered, which could suggest the middle ear as possible predilection site for certain *M. bovis* strains. However, no definitive conclusions can be drawn as there were only few isolates obtained from this isolation site in the present study. In addition, we isolated different *M. bovis* strains (Mb49, Mb50) from two veal calves on the same farm at the same time. The observation of two different strains in one herd or even one animal has been described before (Butler et al., 2001; Soehnlen et al., 2011; Sulyok et al., 2014; Rosales et al., 2015), in contrast to Arcangioli et al. (2012), who isolated only one identical dominant profile in the same feedlot.

Finally, it was evident that Belgian isolates were mostly related to European and Israeli *M. bovis* isolates, even though only a few genomes of European *M. bovis* isolates have been published in the NCBI database. This seems plausible, as Belgian farmers mostly purchase cattle from European farms, while Israel also partly imports cattle from Eastern-Europe. The fact that Israeli isolates are often related to Chinese and Australian strains, is also due to import of cattle, as outlined in detail elsewhere (Menghwar et al., 2017; Yair et al., 2020). Some of the Belgian outlier strains were related to American isolates, which might be explained by the fact that *M. bovis* was first isolated in the USA and outbreaks in Europe were only seen years later. So, we can only speculate whether these outlier strains could have been imported or evolved geographically distinct from each other. Clusters of the Belgian isolates were not clustered exactly in the same way in the Belgian vs. the worldwide phylogenetic tree. A possible explanation could be the loss of overall coverage between the construction of both phylogenetic trees (51% for the Belgian to 40% worldwide). This might be due to (1)

heterogeneity among isolates worldwide and/or (2) the use of genomes obtained by different laboratories, using different sequencing protocols, as the quality can be influenced by strain maintenance, DNA extraction, library preparation, sequencing, and the bioinformatics analysis (Portmann et al., 2018).

In conclusion, multiple *M. bovis* clusters were circulating in Belgium in 2014-2019, and were persisting for several years. Neither the veal industry, nor any other cattle industry could be identified as source of strain persistence. Connections between dairy, beef and veal industry are intense and *M. bovis* appears to easily spread among these sectors. The *M. bovis* issues in the veal industry seem more likely the consequences of strain import from dairy and beef, rather than persistence of a limited number of veal specific strains. This information can contribute to better control and prevention *of M. bovis* infections by improved biosecurity.

AVAILABILITY OF DATA AND MATERIALS

All *M. bovis* consensus genomes are available for download on the NCBI GenBank database under the BioProject PRJNA639688 and accession numbers (SAMN15246515-SAMN1524662). Sequencing summaries can be found in Supplement 1.

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SUPPLEMENTARY DATA

Supplement 1. Sequence identification and descriptives of Belgian *Mycoplasma bovis* isolates), can be found online at https ://doi.org/10.1186/s1356 7-020-00848 -z.

Supplement 2. Sequence identification and descriptives of 250 previously published *Mycoplasma bovis* sequences, can be found online at https ://doi.org/10.1186/s1356 7-020-00848 -z.

REFERENCES

Aebi, M., Bodmer, M., Frey, J., Pilo, P. 2012. Herd-specific strains of *Mycoplasma bovis* in outbreaks of mycoplasmal mastitis and pneumonia. *Vet Microbiol 157*, 363-368.

Arcangioli, M.A., Aslan, H., Tardy, F., Poumarat, F., Le Grand, D. 2012. The use of pulsed-field gel electrophoresis to investigate the epidemiology of *Mycoplasma bovis* in French calf feedlots. *Vet J 192*, 96-100.

Arcangioli, M.A., Duet, A., Meyer, G., Dernburg, A., Bézille, P., Poumarat, F., Le Grand, D. 2008. The role of *Mycoplasma bovis* in bovine respiratory disease outbreaks in veal calf feedlots. *Vet J 177,* 89-93.

Becker, C.A.M., Thibault, F.M., Arcangioli, M.A., Tardy, F. 2015. Loss of diversity within *Mycoplasma bovis* isolates collected in France from bovines with respiratory diseases over the last 35years. *Infect Genet Evol 33*, 118-126.

Bokma, J., Boone, R., Deprez, P., Pardon, B. 2019. Risk factors for antimicrobial use in veal calves and the association with mortality. *J Dairy Sci 102,* 607-618.

Bokma J., Pardon B., Van Driessche L., Gille L., Deprez P., Haesebrouck F., Boyen F. 2019. Optimizing identification of *Mycoplasma bovis* by MALDI-TOF MS. *Research in Veterinary Science 125*, 185-188.

Bokma, J., Van Driessche, L., Deprez, P., Haesebrouck, F., Vahl, M., Weesendorp, E., Deurenberg, R.H., Pardon, B., Boyen, F. 2020. Rapid identification of *Mycoplasma bovis* strains from bovine bronchoalveolar lavage fluid with MALDI-TOF MS after enrichment procedure. *J Clin Microbiol 58*, e00004-20.

Bürki, S., Spergser, J., Bodmer, M., Pilo, P. 2016. A dominant lineage of *Mycoplasma bovis* is associated with an increased number of severe mastitis cases in cattle. *Vet Microbiol 196*, 63-66.

Butler, J.A., Pinnow, C.C., Thomson, J.U., Levisohn, S., Rosenbusch, R.F. 2001. Use of arbitrarily primed polymerase chain reaction to investigate *Mycoplasma bovis* outbreaks. *Vet Microbiol 78,* 175-181.

De Coster, W., D'Hert, S., Schultz, D.T., Cruts, M., Van Broeckhoven, C. 2018. NanoPack: Visualizing and processing long-read sequencing data. *Bioinformatics 34*, 2666-2669.

Deng, X., den Bakker, H.C., Hendriksen, R.S. 2016. Genomic epidemiology: Whole-genome-sequencing–powered surveillance and outbreak investigation of foodborne bacterial pathogens. *Annu Rev Food Sci Technol 7,* 353-374.

Ensoy, C., Faes, C., Welby, S., Van der Stede, Y., Aerts, M. 2014. Exploring cattle movements in Belgium. *Prev Vet Med 116*, 89-101.

Fox, L.K., Kirk, J.H., Britten, A. 2005. Mycoplasma mastitis: A review of transmission and control. *J Vet Med Ser B Infect Dis Vet Public Heal 52*, 153-160.

Gautier-Bouchardon, A. V., Ferré, S., Le Grand, D., Paoli, A., Gay, E., Poumarat, F. 2014. Overall

decrease in the susceptibility of *Mycoplasma bovis* to antimicrobials over the past 30 years in France. *PLoS One 9*, e87672.

Gautier-Bouchardon, A. V. 2018. Antimicrobial Resistance in *Mycoplasma* spp. *Microbiol Spectr 6*.

Gille, L., Pilo, P., Valgaeren, B.R., Van Driessche, L., Van Loo, H., Bodmer, M., Bürki, S., Boyen, F., Haesebrouck, F., Deprez, P., Pardon, B. 2016. A new predilection site of *Mycoplasma bovis:* Postsurgical seromas in beef cattle. *Vet Microbiol* 186, 67-70.

Gonzalez, R.N., Sears, P.M., Merrill, R.A., Hayes, G.L. 1992. Mastitis due to *Mycoplasma* in the state of New York during the period 1972-1990. *Cornell Vet 82,* 29-40.

Goldstein, S., Beka, L., Graf, J., Klassen, J.L. 2019. Evaluation of strategies for the assembly of diverse bacterial genomes using MinION long-read sequencing. *BMC Genomics 20*, 1-17.

Goodwin, S., McPherson, J.D., McCombie, W.R. 2016. Coming of age: Ten years of next-generation sequencing technologies. *Nat Rev Genet 17*, 333-351.

Haapala, V., Pohjanvirta, T., Vähänikkilä, N., Halkilahti, J., Simonen, H., Pelkonen, S., Soveri, T., Simojoki, H., Autio, T. 2018. Semen as a source of *Mycoplasma bovis* mastitis in dairy herds. *Vet Microbiol 216*, 60-66.

Hata, E., Harada, T., Itoh, M. 2019. Relationship between antimicrobial susceptibility and multilocus sequence type of *Mycoplasma bovis* isolates and development of a method for rapid detection of point mutations involved in decreased susceptibility to macrolides, lincosamides, tetracyclines,. *Appl Environ Microbiol 85*, e00575-19.

Kaas, R.S., Leekitcharoenphon, P., Aarestrup, F.M., Lund, O. 2014. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. *PLoS One 9*, e104984.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol 35*, 1547-1549.

Leekitcharoenphon, P., Nielsen, E.M., Kaas, R.S., Lund, O., Aarestrup, F.M. 2014. Evaluation of whole genome sequencing for outbreak detection of *Salmonella* Enterica. *PLoS One 9*, e87991.

Maiden, M.C.J., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., Spratt, B.G. 1998. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci USA 95*, 3140-3145.

Maunsell, F.P., Donovan, G.A. 2009. *Mycoplasma bovis* infections in young calves. *Vet Clin North Am Food Anim Pract 25*, 139-177.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle*. J Vet Intern Med 25*, 772-783.

Maunsell, F.P., Chase, C. 2019. *Mycoplasma bovis*: Interactions with the immune system and failure to generate an effective immune response. *Vet Clin North Am Food Anim Pract 35*, 471-483.

McAuliffe, L., Kokotovic, B., Ayling, R.D., Nicholas, R.A.J. 2004. Molecular epiderniological

analysis of *Mycoplasma bovis* isolates from the United Kingdom shows two genetically distinct clusters. *J Clin Microbiol 42*, 4556-4556.

Menghwar, H., He, C., Zhang, H., Zhao, G., Zhu, X., Khan, F.A., Faisal, M., Rasheed, M.A., Zubair, M., Memon, A.M., Ridley, A., Robertson, I.D., Chen, Y., Guo, A. 2017. Genotype distribution of Chinese *Mycoplasma bovis* isolates and their evolutionary relationship to strains from other countries. *Microb Pathog 111*, 108-117.

Nicholas, R.A.J., Ayling, R.D. 2003. *Mycoplasma bovis*: Disease, diagnosis, and control. *Res Vet Sci 74*, 105-112.

Pardon, B., De Bleecker, K., Dewulf, J., Callens, J., Boyen, F., Catry, B., Deprez, P. 2011. Prevalence of respiratory pathogens in diseased, non-vaccinated, routinely medicated veal calves. *Vet Rec 169,* 278.

Pardon, B. 2012. Morbidity, mortality and drug use in white veal calves emphasis on respiratory disease. PhD Thesis, Ghent University, Faculty of Veterinary Medicine, Department of Large Animal Internal Medicine

Pardon, B., Catry, B., Boone, R., Theys, H., De Bleecker, K., Dewulf, J. Deprez, P. 2014. Characteristics and challenges of the modern Belgian veal industry. *Vlaams Diergeneeskd Tijdschr 83*, 155-163.

Pardon, B., Callens, J., Maris, J., Allais, L., Van Praet, W., Deprez, P., Ribbens, S. 2020. Pathogen-specific risk factors in acute outbreaks of respiratory disease in calves. *J Dairy Sci 103,* 2556-2566.

Parker, Alysia M., Shukla, A., House, J.K., Hazelton, M.S., Bosward, K.L., Kokotovic, B., Sheehy, P.A. 2016. Genetic characterization of Australian *Mycoplasma bovis* isolates through whole genome sequencing analysis. *Vet Microbiol 196*, 118-125.

Portmann, A.C., Fournier, C,. Gimonet, J., Ngom-Bru, C., Barretto, C., Baert, L. 2018. A validation approach of an end-to-end whole genome sequencing workflow for source tracking of *Listeria monocytogenes* and *Salmonella* Enterica. *Front Microbiol 9*, 446.

Quick, J., Ashton, P., Calus, S., Chatt, C., Gossain, S., Hawker, J., Nair, S., Neal, K., Nye, K., Peters, T., De Pinna, E., Robinson, E., Struthers, K., Webber, M., Catto, A., Dallman, T.J., Hawkey, P., Loman, J.N. 2015. Rapid draft sequencing and real-time nanopore sequencing in a hospital outbreak of *Salmonella*. *Genome Biol 16*, 114.

Rang, F.J., Kloosterman, W.P., de Ridder, J. 2018. From squiggle to basepair: Computational approaches for improving nanopore sequencing read accuracy. *Genome Biol 19*, 90.

Register, K.B., Thole, L., Rosenbush, R.F., Minion, F.C. 2015. Multilocus sequence typing of *Mycoplasma bovis* reveals host-specific genotypes in cattle versus bison. *Vet Microbiol 175,* 92-98.

Register, K.B., Jelinski, M.D., Waldner, M., Boatwright, W.D., Anderson, T.K., Hunter, D.L., Hamilton, R.G., Burrage, P., Shury, T., Bildfell, R., Wolff, P.L., Miskimins, D., Derscheid, R.J.,

Woodbury, M.R. 2019. Comparison of multilocus sequence types found among North American isolates of *Mycoplasma bovis* from cattle, bison, and deer, 2007–2017. *J Vet Diagn Invest 31*, 899-904.

Rosales, R.S., Churchward, C.P., Schnee, C., Sachse, K., Lysnyansky, I., Catania, S., Iob, L., Ayling, R.D., Nicholas, R.A.J. 2015. Global multilocus sequence typing analysis of *Mycoplasma bovis* isolates reveals two main population clusters. *J Clin Microbiol 53*, 789-794

Schürch, A.C., Arredondo-Alonso, S., Willems, R.J.L., Goering, R.V. 2018. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene–based approaches. C*lin Microbiol Infect 24*, 350-354.

Soehnlen, MK.,. Kariyawasam, S., Lumadue, J.A., Pierre, T.A., Wolfgang, D.R., Jayarao, B.M. 2011. Molecular epidemiological analysis of *Mycoplasma bovis* isolates from the Pennsylvania Animal Diagnostic Laboratory showing genetic diversity. *J Dairy Sci 94*, 1893-1899.

Sović, I., Šikić, M.,Wilm, A., Fenlon, S.N., Chen, S., Nagarajan, N. 2016. Fast and sensitive mapping of nanopore sequencing reads with GraphMap. *Nat Commun 7*, 11307.

Spergser, J., Macher, K., Kargl, M., Lysnyansky, I., Rosengarten, R. 2013. Emergence, re-emergence, spread and host species crossing of *Mycoplasma bovis* in the Austrian Alps caused by a single endemic strain. *Vet Microbiol 164*, 299-306.

Sulyok, K.M., Kreizinger, Z., Fekete, L., Jánosi, S., Schweitzer, N., Turcsányi, I., Makrai, L., Erdélyi, K., Gyuranecz, M. 2014. Phylogeny of *Mycoplasma bovis* isolates from Hungary based on multi locus sequence typing and multiple-locus variable-number tandem repeat analysis. *BMC Vet Res 10*, 108.

Theuns, S., Vanmechelen, B., Bernaert, Q., Deboutte, W., Vandenhole, M., Beller, L., Matthijnssens, J., Maes, P., Nauwynck, H.J. 2018. Nanopore sequencing as a revolutionary diagnostic tool for porcine viral enteric disease complexes identifies porcine kobuvirus as an important enteric virus. *Sci Rep 8*, 9830.

Wood, D.E., Lu, J., Langmead, B. 2019. Improved metagenomic analysis with Kraken 2. *Genome Biol 20*, 257.

Yair, Y., Borovok, I., Mikula, I., Falk, R., Fox, L.K., Gophna, U., Lysnyansky, I. 2020. Genomics-based epidemiology of bovine *Mycoplasma bovis* strains in Israel. *BMC Genomics 21*, 70

CHAPTER 5

ANTIMICROBIAL SUSCEPTIBILITY OF MYCOPLASMA BOVIS

ANTIMICROBIAL SUSCEPTIBILITY OF *MYCOPLASMA BOVIS* ISOLATES FROM VEAL, DAIRY AND BEEF HERDS

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ABSTRACT

Mycoplasma bovis is an important pathogen causing mostly pneumonia in calves and mastitis in dairy cattle. In absence of an effective vaccine, antimicrobial therapy remains the main control measure. Antimicrobial use in veal calves is substantially higher than in conventional herds, but whether veal calves also harbour more resistant *M. bovis* strains, is currently unknown. Therefore, we compared antimicrobial susceptibility test results of *M. bovis* isolates from different cattle sectors and genomic clusters. The minimum inhibitory concentration of 9 antimicrobials was determined for 141 Belgian *M. bovis* isolates (29 dairy, 69 beef, 12 mixed, 31 veal farms), and used to estimate the epidemiological cut-off. Acquired resistance was frequently observed for the macrolides, while no acquired resistance to oxytetracycline and doxycycline, minimal acquired resistance to florfenicol and tiamulin, and a limited acquired resistance to enrofloxacin was seen. *M. bovis* isolates from beef cattle or genomic cluster III had higher odds to be gamithromycin resistant than those from dairy cattle or genomic clusters IV and V. In this study, no cattle industry could be identified as source of resistant *M. bovis* strains. A single guideline irrespective of cattle sector for antimicrobial use for *M. bovis* infections, with a small remark for gamithromycine, is likely sufficient.

Keywords: epidemiological cut-off methods; gamithromycin; genomic clusters; iterative statistical method; normalized resistance interpretation; visual estimation

INTRODUCTION

In the last decade, *Mycoplasma bovis (M. bovis)* has come to the forefront as an economically important bacterium with a large impact on health, welfare and antimicrobial use (AMU) in cattle operations worldwide (Calcutt et al., 2018). The bacterium is mainly feared as the cause of pneumonia, arthritis and otitis in calves, and pneumonia and mastitis in adult cattle (Maunsell and Donovan, 2009; Maunsell et al., 2011). In the absence of an effective vaccine, antimicrobial therapy remains a crucial factor to control an outbreak.

In recent years, a decrease in antimicrobial susceptibility of *M. bovis* to various antimicrobial classes targeting protein synthesis (e.g. phenicols, tetracyclines, lincosamides and macrolides) and DNA synthesis (e.g. fluoroquinolones) has been reported in different countries (Gautier-Bouchardon et al., 2014; Cai et al., 2019; Klein et al., 2019; García-Galán et al., Jelinski et al., 2020; Liu et al., 2020). Especially for macrolides high percentages of resistant *M. bovis* isolates are reported, while fluoroquinolones remain the most effective antimicrobial *in vitro* in most countries (Heuvelink et al., 2016; Cai et al., 2019; Becker et al., 2020; Liu et al., 2020), except for Spain and Italy (Klein et al., 2019; García-Galán et al., 2020). Geographical differences in antimicrobial susceptibility of *M. bovis,* as well as differences between strains isolated from various predilection sites, such as the joint, udder and lung have been described (Gerchman et al., 2009; Heuvelink et al., 2016; Cai et al., 2019). Also more antimicrobial resistance was observed in Canadian *M. bovis* strains obtained from dead animals compared to those isolated from healthy animals (Jelinski et al., 2020). Despite that antimicrobial susceptibility differences between production systems have been shown for other respiratory pathogens, such as *Pasteurella* and *Mannheimia* isolates (Catry et al., 2005), this was not previously explored for *M. bovis*. Quantity and quality of AMU can differ greatly between production systems, with veal calf operations showing much higher AMU compared to conventional herds (Dorado-García et al., 2016). Varying AMU may result in a different resistance selection pressure, subsequently changing antimicrobial susceptibility patterns or supporting clonal emergence of specific *M. bovis* strains in outbreaks (Becker et al., 2015; García-Galán et al., 2020; Liu et al., 2020). If production-specific antimicrobial susceptibility exists for *M. bovis*, it might be necessary to adjust *M. bovis* treatment guidelines to specific production systems. Also, whether antimicrobial resistance is associated with specific genetic strains is not clear, as some studies observed no association between genetic subtypes based on the *polC* subtyping scheme (Becker et al., 2020), while others did for lincosamides and macrolides (Liu et al., 2020). With whole genome sequencing (WGS) becoming more popular

and commercially available for identification and strain typing (Parker et al., 2016; Yair et al., 2020; Bokma et al., 2020a), it might be helpful to determine whether phenotypic antimicrobial susceptibility patterns are associated with genomic clusters of *M. bovis*. Therefore, the objective of the present study was to compare antimicrobial susceptibility results of *M. bovis* isolates obtained from veal calf, conventional dairy and beef herds, and to explore the association of AMR with specific *M. bovis* genomic clusters.

MATERIAL AND METHODS

Mycoplasma bovis collection

One hundred forty-one epidemiologically independent *M. bovis* isolates, originating from 29 dairy, 69 beef, 12 mixed (both dairy and beef) and 31 veal farms were included in this study. Isolates were obtained from the respiratory tract (128), middle ear (4), milk (5), joint (2), abscess (1) and seroma (1), collected in Belgium between 2016 and 2019, with the exception of one isolate which was obtained in 2014. One hundred of these isolates have been strain typed previously [18]. All isolates were obtained from diagnostic samples collected by field veterinarians from clinical cases, in compliance with EU legislation on ethics in animal experimentation [2010/63/EU]. All samples were cultured on a modified pleuropneumonia-like organism (PPLO) agar plate and incubated for 7-10 days (37°C, 5% CO_2). Presumptive *M. bovis* identification was based on the typical fried-egg colony appearance on modified PPLO agar and the presence of lipase activity as tested on medium containing Tween-80 (Bokma et al., 2020b). Final identification was performed with MALDI-TOF MS as described before (Bokma et al., 2019). All samples were stored at -80°C until further analysis.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) were obtained following the guidelines for MIC testing of veterinary *Mycoplasma* spp. described by Hannan (2000). *M. bovis* isolates were thawed and cultured on modified PPLO agar. After 7 days of incubation at 37°C in a 5% CO_2 enriched atmosphere, colonies were inoculated in modified PPLO broth (pH 7.8) (Difco™, BD Diagnostic Systems, Sparks, Md.) supplemented with 25% inactivated horse serum (Gibco™), 0.7% technical yeast extract, 0.5% sodium pyruvate (ReagentPlus, Sigma-Aldrich®), 0.5% D-(+)-glucose monohydrate (Sigma-Aldrich) and 0.005% phenol red as growth indicator. After 3 days of incubation (37°C; 5% CO_2) a bacterial suspension of approximately 10^8 CFU/ml was obtained. Ten-fold serial dilutions were made using the same

broth, and 200 µl of the diluted suspension with approximately 10^5 CFU/ml was transferred to each well of a custom-made 96-U-bottom well Sensititre microplate (Thermofisher) containing doubling florfenicol concentrations between 0.25 and 128 µg/ml, oxytetracycline (0.12-128 µg/ml), doxycycline (0.06-32 µg/ml), tilmicosin (0.06-128 µg/ml), tylosin (0.06-32 µg/ml), gamithromycin (0.06-256 µg/ml), tiamulin (0.03-1 µg/ml), gentamicin (0.06-32 µg/ml) and enrofloxacin (0.06-32 µg/ml). Additionally, two growth control wells (no antimicrobial, with inoculum), a sterility control well (200 µL uninoculated broth, pH 7.8) and a pH control well (200 µL uninoculated broth, pH 6.8) were added to each plate. Hundred µL from one growth control well was used to perform ten-fold dilutions and subsequent inoculation on a modified PPLO agar plate. Colonies were counted after 7 days of incubation at 37°C in a 5% CO_2 enriched atmosphere, to confirm inoculum concentrations were within a 10^3-10^5 CFU/ml range.

Plates were sealed with adhesive foil and incubated at 37°C. Interpretation of color change from red to orange/yellow was done after 48 and 72 hours of incubation. The lowest antimicrobial concentration without color change was recorded as the MIC at the earliest time point the growth control well had the same color as the pH control well. When for a certain isolate, more than one skipped well was observed, results for these isolates were excluded. When only one well was skipped, the highest MIC value was listed (EUCAST, 2020). Quality control was performed in every run (six in total) by determining the MIC values of *M. bovis* strain PG45 (ATCC 25523) and comparing these to previously published values (Ter Laak et al., 1993; Gerchman et al., 2009; Sulyok et al., 2014; García-Galán et al., 2020). Reference strains *Staphylococcus aureus* (ATCC®29213™) and *Escherichia coli* (ATCC®25922™) were included as extra quality control strains in the same broth as *M. bovis*, but observed after 24 hours of incubation.

Interpretation of MIC values

Due to the lack of clinical breakpoints (CBPs), the interpretation of MIC values of *M. bovis* is not straightforward (Rosenbusch et al., 2005; Maunsell et al., 2001; Toutain et al., 2017). The best option to interpret *M. bovis* MIC data is probably to determine the epidemiological cutoff value (ECOFF). With this method, wild-type bacterial populations are distinguished from those with acquired resistance (non-wild type) by observing the MIC distribution. Three methods to estimate the ECOFF were compared in this study: the visual estimation ("eyeball") method based on the uni-, bi- or multimodal MIC distribution and/or tailing, as described previously (Turndige et al., 2007; Toutain et al., 2017) and two statistical methods:

"Normalized Resistance Interpretation (NRI)" (http://www.bioscand.se/nri/, Bioscand AB, Täby, Sweden; Callens et al., 2016) and the "Iterative Statistical Method" processed in ECOFFinder (version 2.1; https://www.eucast.org/mic_distributions_and_ecoffs/, EUCAST) (Turnidge et al., 2006; Kronvall et al., 2010). Instructions provided by the founders were followed. When using the NRI method, standard deviations of the normal distribution of wild type MIC values exceeding 1.2 $\log_2$, result in a tentative estimate of the ECOFF and one can only speak of the "putative wild type group". With ECOFFinder plots for residuals were checked and categorized, corresponding to whether the residuals scattered on either side of the horizontal axis in the center (well fit, selected subset values are considered reliable), only partly (poor fit) or not (no fit). As users can choose the cut-off value (95% to 99.9%) with ECOFFinder, depending on the intended use and influencing the sensitivity and specificity of the (non-)wild type population, both the 95% and 99% cut-off were determined. In addition, the MIC_{50} and MIC_{90} were calculated as the lowest MIC at which at least 50% and 90% of the isolates in a test population are inhibited in their growth, respectively. Since no ECOFF within the testing range of our study could be obtained by any of the three methods for tilmicosin, also previously published data and cross-resistance with tylosin were taken into account to make the decision that the isolates with MIC ≥ 32 µg/ml belonged to the non-wild type population (see discussion section). The latter tilmicosin ECOFF, together with the ECOFFs obtained with the visual estimation method, were used in further analysis to compare AMR in *M. bovis* isolates obtained from different cattle sectors or belonging to specific genomic clusters.

Statistical analysis

To determine whether there are significant differences between conventional herds (dairy, beef) and veal calves, a logistic regression was performed on binary variables, representing acquired resistance (1) and wild type (0) isolates. A *P*-value smaller than 0.05 was considered statistically significant. The Hosmer-Lemeshow test was included to determine the goodness of fit of the model (SPSS Statistics 26). To allow a meaningful statistical analysis, only for antimicrobials for which 5 to 95% acquired resistance was observed, statistical analysis was done.

In a former study, a subset of 100 isolates of the currently used *M. bovis* database has been strain typed, as described earlier [18]. These isolates have been categorized in 5 phylogenetic clusters, based on single nucleotide polymorphism (SNP) analysis (Bokma et al., 2020a). In the present study, the association between the presence of acquired resistance according to the

visual estimation method and the phylogenetic clusters of this subset of strains was investigated for antimicrobials for which 5 to 95% acquired resistance was observed, and further visualized in MEGA-X (Kumar et al., 2018). Logistic regression on binary variables (1: acquired resistance; 0: wild type) was only performed for cluster III to V, as cluster I and II did not contain enough *M. bovis* isolates for the model to run.

RESULTS

Antimicrobial susceptibility of Belgian *M. bovis*

MIC results of 141 epidemiologically unrelated *M. bovis* isolates are shown in Table 1. All obtained isolates were identified as *M. bovis* with MALDI-TOF MS (score value ≥ 1.7). MIC values for the *M. bovis* PG45 reference strain were within a small range of dilutions between runs for gamithromycin (8-16 µg/ml), tilmicosin (0.12-0.5 µg/ml), florfenicol (1-2 µg/ml), doxycycline (≤ 0.06-0.12 µg/ml), enrofloxacin (≤ 0.06-0.12 µg/ml), tylosin (≤ 0.06-0.12 µg/ml), tiamulin (≤ 0.03 µg/ml), and oxytetracycline (≤ 0.12 µg/ml) and were similar to previously described results (Ter Laak et al., 1993; Rosenbusch et al., 2005; Gerchman et al., 2009; Sulyok et al., 2017; Heuvelink et al., 2016; García-Galán et al., 2020). The quality control (QC) MIC-values for *Staphylococcus aureus* subsp. aureus (ATCC®29213™) and *Escherichia coli* (ATCC®25922™) were within the acceptable quality control ranges as provided by the Sensititre manufacturer. Results for gentamicin were excluded from this study, as the quality control was not passed, and MIC values for type strain *M. bovis* PG45 deviated from previous studies (Gerchman et al., 2009; Sulyok et al., 2014). However, no quality control strains were included in previous studies, therefore the results are included in Supplementary file 1.

Interpretation of MIC values

The ECOFF as determined using the different methods and percentage of isolates belonging to the wild type and non-wild type population are shown in Table 2. For all three ECOFF methods, acquired resistance was frequently observed for macrolides (gamithromycin, tylosin), while only a few isolates showed acquired resistance against florfenicol, enrofloxacin and tiamulin. Following the visual estimation method, no acquired resistance against oxytetracycline and doxycycline was observed. Although, the statistical methods categorized part of the population (3.6%-13.0%) as non-wild type for doxycycline.

Table 1. Distribution of MIC-values (µg/ml) of 141 *M. bovis* isolates obtained from cattle in Belgium over 2016 - 2019

		Distribution of MICs (µg/ml)														
Class	Antimicrobial	≤ 0.03	≤ 0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	Total
Phenicol	Florfenicol	ND	ND	ND	1	5	27	44	43	13	3	4			ND	140
Tetracycline	Oxytetracycline	ND	ND	5	2	15	49	46	17	5					ND	139
	Doxycycline	ND	3	4	43	47	23	13	5				ND	ND	ND	138
Macrolide	Tilmicosin	ND								1			3	23	114	141
	Tylosin	ND					1	3	11	16	22	7	77[a]	ND	ND	137
	Gamithromycin	ND	1				3	2	8	19	26	9	1	4	66	139
Pleuromutilin	Tiamulin	46	43[c]	29	15	5			1[b]	ND	ND	ND	ND	ND	ND	139
Fluoroquinolone	Enrofloxacin	ND	1	17	51	37	20	2	3	2	2	1	1[a]	ND	ND	137

ND: not determined, [a] MIC ≥ 32, [b] MIC ≥ 2, [c] MIC 0.06; ECOFFs based on the visual estimation method are shown as black vertical lines

We were able to determine the ECOFF for 7 out of the 8 antimicrobials with the visual estimation method, whereas normalized resistance interpretation (NRI) and ECOFFinder determined a reliable ECOFF in 8/8 and 3/8 of the antimicrobials, respectively. The NRI method was able to determine an ECOFF for every antimicrobial tested, even when this needed extrapolation from the tested MIC range, such as for tilmicosin. However, when the standard deviation of the normal distribution of the wild type MIC value is > 1.2 $\log_2$, the program provides only the "putative wild type population", as was the case for the macrolides. The ECOFFinder method was only able to determine reliable results (good fit plots for residuals), for florfenicol and the two tetracyclines. Truncated distributions influence the reliability or possibility to interpret some of the results, such as those for the macrolides. All three methods determined ECOFF values for corresponding antimicrobials within one dilution, except for doxycycline and tiamulin. This results in substantial differences in the (non)wild-type population. When ECOFFinder 95% was used for doxycycline, 13% was categorized as non-wild type in comparison to 0% when using the visual estimation method. For tiamulin the visual method indicated 0.7% non-wild type, whereas NRI indicated 15.1%. This might be due to the combination of "tailing" and the lack of a normal distribution, which complicates the interpretation of the MIC distributions, both visually and statistically.

Table 2. Epidemiological cut-offs for *M. bovis* from Belgian cattle (n = 141) based on the visual estimation method, NRI and with ECOFFinder, resulting in different percentages of wild type (WT) and non-wild type (n-WT)

Class	Antimicrobial	Visual estimation	WT (%)	n-WT (%)	NRI	WT (%)	n-WT (%)	ECOFFinder (95%/99%)*	WT (%)	n-WT (%)
Phenicol	Florfenicol	> 16	97.1	2.9	> 16	97.1	2.9	> 8/16 (+)	95.0/97.1	5.0/2.9
Tetracycline	Oxytetracycline	> 8	100.0	0.0	> 8	100.0	0.0	> 4/8 (+)	96.4/100.0	3.6/0.0
	Doxycycline	> 4	100.0	0.0	> 2	96.4	3.6	> 1/2 (+)	87.0/96.4	13.0/3.6
Macrolide	Tilmicosin	ND	-	-	> 1024	-	-	ND	-	-
	Tylosin	> 32	43.8	56.2	> 128 #	-	-	ND	-	-
	Gamithromycin	> 64	49.6	50.4	> 128 #	52.5	47.5	ND	-	-
Pleuromutilin	Tiamulin	> 0.5	99.3	0.7	> 0.125	84.9	15.1	> 0.06/0.06 (-)	-	-
Fluoroquinolone	Enrofloxacin	> 2	93.4	6.6	> 1	92.0	8.0	> 1/2 (±)	92.0/93.4	8.0/6.6

= tentative estimate, as standard deviation > 1.2 $\log_2$

* plots for residuals were checked and categorized in either well fit (+), poor fit (±) or no fit (-) corresponding to whether the subset values are reliable or not. ND: not possible to determine

Variability of antimicrobial susceptiblity per production system

The distribution of *M. bovis* MIC values for the different antimicrobials and per production system are available in Supplementary file 2. Logistic regression did not show significant differences in antimicrobial resistance between production systems, except for gamithromycin (Table 3). Beef *M. bovis* isolates (58.21% acquired resistance) had a three times higher odds (CI 95%: 1.23-7.69) for gamithromycin resistance than dairy isolates (31.03%; $P = 0.02$).

The MIC_{50} and MIC_{90} values are shown per sector in Table 4. No differences in MIC_{50} were observed between sectors for tilmicosin, doxycycline, and tiamulin. A single two-fold dilution difference in MIC_{50} between herd types was observed for florfenicol (highest in veal), oxytetracycline (lowest in dairy) and enrofloxacin (lowest in beef). A difference between herds was seen for gamithromycin and to a lesser extend for tylosin. MIC_{50} of gamithromycin was higher in beef cattle (> 128 µg/ml) than in dairy (16 µg/ml) or veal calf isolates (32 µg/ml). No difference was observed in MIC_{90} for florfenicol, tilmicosin, and gamithromycin. While a single two-fold dilution was observed in MIC_{90} for oxytetracycline (lowest in dairy), doxycycline (highest in beef), tylosin (lowest in dairy), tiamulin (lowest in veal) and enrofloxacin (highest in dairy).

Table 3. Results of logistic regression of antimicrobial resistant *M. bovis* isolates obtained from beef, dairy and veal calves over 2016-2019 in Belgium

		ECOFF	WT (%)	nWT (%)	OR	CI95%	*P* - value
Florfenicol	Beef	> 16	95.59	4.41			
	Dairy	> 16	96.55	3.45			
	Veal	> 16	100.00	0.00			
Oxytetracycline	Beef	> 8	100.00	0.00			
	Dairy	> 8	100.00	0.00			
	Veal	> 8	100.00	0.00			
Doxycycline	Beef	> 4	100.00	0.00			
	Dairy	> 4	100.00	0.00			
	Veal	> 4	100.00	0.00			
Tylosin	Beef	> 32	42.42	57.58	Ref		0.48
	Dairy	> 32	51.62	48.38	0.60	(0.25-1.44)	0.25
	Veal	> 32	41.94	58.06	1.02	(0.43-2.42)	0.96
Tilmicosin	Beef	> 32	1.45	98.55			
	Dairy	> 32	0.00	100.00			
	Veal	> 32	0.00	100.00			
Gamithromycin	Beef	> 64	41.79	58.21	Ref		0.06
	Dairy	> 64	68.97	31.03	0.32	(0.13-0.81)	0.02
	Veal	> 64	51.61	48.39	0.67	(0.29-1.58)	0.36
Tiamulin	Beef	> 0.5	98.53	1.47			
	Dairy	> 0.5	100.00	0.00			
	Veal	> 0.5	100.00	0.00			
Enrofloxacin	Beef	> 2	92.54	7.46	Ref		0.98
	Dairy	> 2	92.59	7.41	0.99	(0.18-5.45)	0.99
	Veal	> 2	93.55	6.45	0.86	(0.16-4.67)	0.86

OR = odds ratio; CI95%: 95% confidence interval

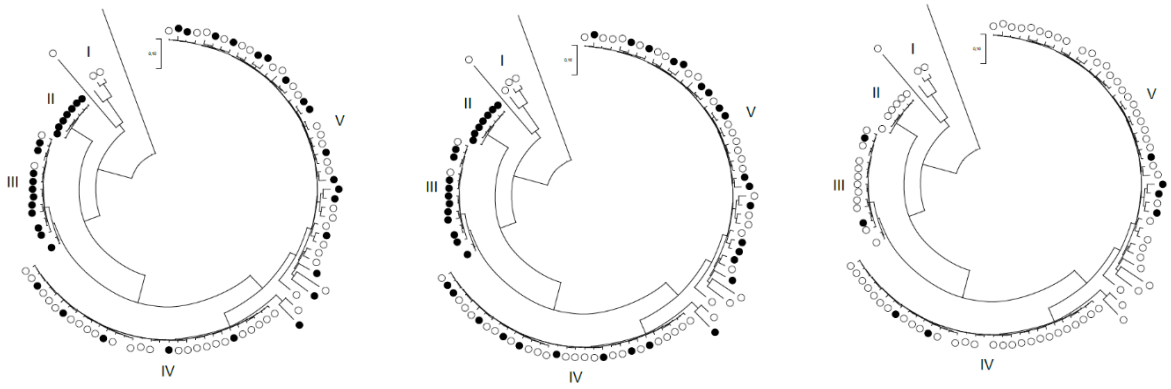
Table 4. MIC_{50} and MIC_{90} (µg/ml) of all *M. bovis* isolates and per sector, obtained from cattle in Belgium over 2016-2019

		Total (n = 144)		Beef (n = 70)		Dairy (n = 31)		Veal (n = 32)	
Class	Antimicrobial	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Phenicol	Florfenicol	2	8	2	8	2	8	4	8
Tetracycline	Oxytetracycline	1	4	2	4	1	2	2	4
	Doxycycline	0.5	2	0.5	2	0.5	1	0.5	1
Macrolide	Tilmicosin	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128
	Tylosin	> 32	> 32	> 32	> 32	16	32	> 32	> 32
	Gamithromycin	128	> 128	> 128	> 128	16	> 128	32	> 128
Pleuromutilin	Tiamulin	0.06	0.25	0.06	0.25	0.06	0.25	0.06	0.12
Fluoroquinolone	Enrofloxacin	0.5	1	0.25	1	0.5	2	0.5	1

MIC_{50}: the lowest MIC at which at least 50% of the isolates were inhibited in their growth MIC_{90}: the lowest MIC at which at least 90% of the isolates were inhibited in their growth

Association between AMR and genomic cluster

In Figure 1, the distribution of the wild type and non-wild type *M. bovis* isolates for gamithromycin, tylosin, and enrofloxacin are shown over the five clusters obtained by whole genome sequencing. Most of the macrolide resistant isolates were located in cluster II and III, whereas cluster I contained isolates susceptible to all antimicrobials. Statistical analysis showed that *M. bovis* isolates from cluster III (85% acquired resistance) had a 22.7 (CI95%: 4.0-125.0, $P < 0.01$) and 7.9 (CI95%: 1.5-40.0, $P = 0.01$) higher odds for gamithromycin resistance compared to cluster IV (19%) and V (41%), respectively. No significant association was found for tylosin or enrofloxacin.



(a) (b) (c)

Figure 1. Phylogenetic tree with the distribution of Belgian *M. bovis* isolates being susceptible (○) or resistant (●) for gamithromycin (a), tylosin (b), and enrofloxacin (c) based on the visual estimation method. These isolates were classified in five genomic clusters by a SNP-analysis of 100 Belgian *M. bovis* isolates (Bokma et al., 2020a). The figure was created using MEGA-X software with *M. bovis* isolates obtained over 2014-2019.

DISCUSSION

In this study, susceptibility of 141 *M. bovis* isolates obtained from beef, dairy and veal calves, was tested against 9 antimicrobial agents covering the 6 antimicrobial classes most frequently used to control *M. bovis* in Belgium, though results of gentamicin (covering the aminoglycosides) were excluded. Since neither clinical breakpoints (CBPs) nor guidelines to interpret ECOFFs for *M. bovis* are available, three methods (visual, NRI and ECOFFinder) to determine ECOFF and interpret antimicrobial susceptibility of *M. bovis* were explored.

Although one should be cautious in comparing results of different studies, because of the lack of internationally recognized standard protocols, the observed MIC values and distributions in this study were similar to previous publications from Western-Europe (Thomas et al., 2003; Barberio et al., 2014; Heuvelink et al., 2016; Klein et al., 2019). A large number of isolates had high MIC values for macrolides, resulting in more than half of the isolates being non-wild type for gamithromycin or tylosin. Cross-resistance between tylosin and tilmicosin has been described for *M. bovis* (Lerner et al., 2014; Sulyok et al., 2017), but determination of ECOFF based on the MIC values for tilmicosin was not possible as no normal distribution was obtained due to the very high MIC values.

Surprisingly, there were no indications for acquired resistance against tetracyclines. Current study showed decreased MIC_{50-90} values (1-4 µg/ml) of *M. bovis* for oxytetracycline compared with a 20-year old Belgian study reporting MIC_{50-90} values of 2 and 32 µg/ml respectively (Thomas et al., 2003). In other European countries as well, a similar trend has been observed in recent years (Heuvelink et al., 2016; Klein et al., 2019). For doxycycline, percentages of acquired resistance depended on the ECOFF method used (0% to 13%). When using the 95% rule with ECOFFinder, 13% acquired resistance was obtained, while using the 99% rule only 3.6% resistant isolates were observed. One should be aware that the decision using 95% or 99% can influence the outcome by either increasing the sensitivity for nWT (95%) or specificity for the WT population (99%). The distribution and MIC_{50-90} for florfenicol were similar (2-8 µg/ml) to previously published data (Gautier-Bouchardon et al., 2014; Heuvelink et al., 2016; Klein et al., 2019), and only four isolates showed acquired resistance (MIC 32 µg/ml). In general, a small non-wild type population was observed for tiamulin. In Europe, pleuromutilins are not registered for use in cattle, while this class of antimicrobials is registered for treatment of *Mycoplasma* infections in pigs and poultry. In addition, valnemulin appears to be very effective against *M. bovis in vivo* (Stipkovits et al., 2005) and low numbers of acquired resistance have been reported in *M. bovis* isolates in France, Spain, and Hungary as well (Gautier-Bouchardon et al., 2014; Sulyok et al., 2017; García-Galán et al., 2020). One possible non-wild type isolate for tiamulin was identified in this study. In a previous study, all tiamulin-resistant mutant strains showed cross-resistance against florfenicol (Sulyok et al., 2017), which was not observed in the current study. Distribution and MIC_{50-90} values for enrofloxacin (0.5-1 µg/ml) were comparable with previous studies (Thomas et al., 2003; Gautier-Bouchardon et al., 2014; Heuvelink et al., 2016), except for Klein et al. (2019), who found higher MIC_{90} values (8 µg/ml).

Gentamicin did not pass the quality control with *S. aureus* (ATCC 29213) and *E. coli* (ATCC 25922), also MIC for *M. bovis* PG45 deviated from previous studies (Gerchman et al., 2009; Sulyok et al., 2014), based on these observations the results were excluded. Previous studies did not include these quality control strains, and therefore we are not aware whether this is a reoccurring problem (Thomas et al., 2003; Gerchman et al., 2009; Sulyok et al., 2014). It is likely that specific medium components, resulting in an adjusted pH, have altered the results of the quality control strains (Gudmundsson et al., 1991), but as there is no standard protocol or quality control standard for *M. bovis* PG45, more research is necessary. Notwithstanding this, we included the results in the supplementary data to contribute to this research (Supplementary file 1).

We did not observe significant sector-specific antimicrobial resistance, except for gamithromycin. *M. bovis* isolates from beef cattle had higher odds to be non-wild type than those from dairy cattle. Dairy cattle isolates also had the lowest MIC_{50} for tylosin. This could possibly be explained by the low number of macrolides registrated for use in lactating animals, and the high use of macrolides to combat bovine respiratory disease in beef cattle and veal calves. Also other factors, such as age, housing conditions and milk diet could play a part in the evolution of antimicrobial resistance in different production systems (Catry et al., 2003). Considering the small difference in AMR over sectors, together with previously obtained knowledge about the lack of sector-specific *M. bovis* strains in Belgium (Bokma et al., 2020a), a single guideline for antimicrobial use for *M. bovis* infections covering all different cattle sectors in Belgium, with a small remark for gamithromycine, is likely sufficient.

All used methods to determine the ECOFF are in some way based on a normal distribution. As a consequence, problems occurred with truncated MIC distributions (e.g. tilmicosin, tiamulin). Although the NRI method was able to determine more ECOFFs than the ECOFFinder (only florfenicol and tetracyclines) in an objective manner, the visual estimation method was mostly in agreement with these methods. Even though more subjective, the visual estimation method has the advantage that expert opinion and additional information from MIC data obtained from other class representatives or previous reports can be taken into account. For instance, even though a bimodal distribution was observed for tylosin, the population showing the lower MIC values might not represent the true wild type population. This is supported by a previous MIC study showing a much lower ECOFF (2 μg/ml), while similar QC values were obtained (Klein et al., 2017). In addition, it has been previously shown that specific mutations associated with macrolide resistance were absent in isolates

with MIC values between <0.5 and 4 µg/ml (Lerner et al., 2014). Therefore, an overall shift from *M. bovis* wild type to non-wild type for tylosin is suspected. The same line of reasoning is applicable to tilmicosin. Lerner et al. (2014) did not find any mutations associated with macrolide resistance in isolates with tilmicosin MIC values between < 0.5 and 32 µg/ml. Therefore it seems that all the isolates in this study acquired resistance to tilmicosin to some extent, except for one isolate with a MIC of 1 µg/ml for tylosin and 8 µg/ml for tilmicosin probably representing the wild type population for 16-ring macrolides. Indeed, a recent study showed very high MIC values (≥ 256 µg/ml) for >80% of the *M. bovis* population against tilmicosin (Heuvelink et al., 2016), whereas an older study showed a large population with lower MIC values between 0.5 and 32 µg/ml (Rosenbusch et al., 2005).

Finally, we observed an association between gamithromycin susceptibility patterns and previously published genomic clusters. *M. bovis* isolates in cluster II and III were more frequently belonging to the non-wild type population, than those in cluster IV and V. This might be due to the higher heterogeneity in cluster IV and V caused by genetic drift (Bokma et al., 2020a). Yet, we should be careful in our conclusions, as we are not aware of the influence of genetic drift within any of the cluster on antimicrobial susceptibility data. We believe to this point that even when strain typing can be done very fast and easily, this should always be supplemented with phenotypically antimicrobial susceptibility testing (AST) to detect acquired resistance in *M. bovis* outbreaks. Nevertheless, strain typing could support in the surveillance of AMR by pointing out whether isolates are clonally spread or (closely) related to each other.

CONCLUSIONS

High acquired resistance percentages of *M. bovis* in Belgium were observed for macrolides, with almost all isolates having acquired resistance to 16-ring macrolides and a large proportion to 15-ring macrolides. In addition, minimal acquired resistance to florfenicol and tiamulin was observed, a limited acquired resistance to enrofloxacin, and almost none to the tetracyclines. Higher AMR for gamithromycin was observed in beef cattle compared to dairy, but the veal industry could not be identified as a reservoir of resistant *M. bovis* strains. A single guideline for antimicrobial use for *M. bovis* infections covering all different cattle sectors in Belgium, with a small remark for gamithromycine, is likely sufficient. In addition, only *M. bovis* strains belonging to cluster II and III had more isolates with acquired resistance

for gamithromycin compared to IV and V. Therefore, this study shows that strain typing cannot replace phenotypically AST of *M. bovis* in surveillance programs.

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SUPPLEMENTARY DATA

Supplement 1. Results for gentamicin, can be found online at https://www.mdpi.com/2079-6382/9/12/882

Supplement 2. Distribution of MIC values per sector (beef, dairy, veal), can be found online at https://www.mdpi.com/2079-6382/9/12/882

REFERENCES

Barberio, A., Flaminio, B., De Vliegher, S., Supré, K., Kromker, V., Garbarino, C., Arrigoni, N., Zanardi, G., Bertocchi, L., Gobbo, F., Catania, S., Moroni, P. 2016. Short communication: In vitro antimicrobial susceptibility of *Mycoplasma bovis* isolates identified in milk from dairy cattle in Belgium, Germany, and Italy. *J Dairy Sci 99*, 6578-6584.

Becker, C.A.M., Ambroset, C., Huleux, A., Vialatte, A., Colin, A., Tricot, A., Arcangioli, M.A., Tardy, F. 2020. Monitoring *Mycoplasma bovis* diversity and antimicrobial susceptibility in calf feedlots undergoing a respiratory disease outbreak. *Pathogens 9*, 593.

Becker, C.A.M., Thibault, F.M., Arcangioli, M.A., Tardy, F. 2015. Loss of diversity within *Mycoplasma bovis* isolates collected in France from bovines with respiratory diseases over the last 35years. *Infect Genet Evol 33*, 118-126

Bokma, J., Pardon, B., Van Driessche, L., Gille, L., Deprez, P., Haesebrouck, F., Boyen, F. 2019. Optimizing identification of *Mycoplasma bovis* by MALDI-TOF MS. *Res Vet Sci 125*, 185-188.

Bokma, J., Vereecke, N., De Bleecker, K., Callens, J., Ribbens, S., Nauwynck, H., Haesebrouck, F., Theuns, S., Boyen, F., Pardon, B. 2020a. Phylogenomic analysis of *Mycoplasma bovis* from Belgian veal, dairy and beef herds. *Vet Res 51*, 121.

Bokma, J., Van Driessche, L., Deprez, P., Haesebrouck, F., Vahl, M., Weesendorp, E., Deurenberg, R.H., Pardon, B., Boyen, F. 2020b. Rapid identification of *Mycoplasma bovis* from bovine bronchoalveolar lavage fluid with MALDI-TOF MS after enrichment procedure. *J Clin Microbiol 58*, e00004-20.

Cai, H.Y., McDowall, R., Parker, L., Kaufman, E.I., Caswell, J.L. 2019. Changes in antimicrobial susceptibility profiles of *Mycoplasma bovis* over time. *Can J Vet Res 83*, 34-41.

Calcutt, M.J., Lysnyansky, I., Sachse, K., Fox, L.K., Nicholas, R.A.J., Ayling, R.D. 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. *Transbound Emerg Dis 65,* 91-109.

Callens, B., Dewulf, J., Kronvall, G., Catry, B., Haesebrouck, F., Boyen, F. 2016. Antimicrobial resistance surveillance in *Escherichia coli* by using normalized resistance interpretation. *Vet Microbiol 197,* 1-7.

Garrod, L.P. Waterworth, P.M. 1969. Effect of medium composition on the apparent sensitivity of *Pseudomonas aeruginosa* to gentamicin. *J Clin Pathol 22*, 534-538.

Catry, B.; Laevens, H.; Devriese, L.A.; Opsomer, G.; De Kruif, A. 2003. Antimicrobial resistance in livestock. *J. Vet. Pharmacol. Ther. 26*, 81-93.

Catry, B., Haesebrouck, F., De Vliegher, S., Feyen, B., Vanrobaeys, M., Opsomer, G., Schwarz, S., De Kruif, A. 2005. Variability in acquired resistance of *Pasteurella* and *Mannheimia* isolates from the nasopharynx of calves, with particular reference to different herd types. *Microb Drug Resist*

11, 387-394.

Dorado-García, A., Mevius, D.J., Jacobs, J.J.H., Van Geijlswijk, I.M., Mouton, J.W., Wagenaar, J.A., Heederik, D.J. 2016. Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the Netherlands. *J Antimicrob Chemother 71,* 3607-3619.

EUCAST reading guide for broth microdilution (version 2.0). Available online: https://ww. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2020_manuals/Reading_guide_BMD_v_2.0_2020.pdf (accessed on 17 November 2020)

García-Galán, A., Nouvel, L.X., Baranowski, E., Gómez-Martín, Á., Sánchez, A., Citti, C., de la Fe, C. 2020. *Mycoplasma bovis* in Spanish cattle herds: Two groups of multiresistant isolates predominate, with one remaining susceptible to fluoroquinolones. *Pathogens 9*, 545.

Gautier-Bouchardon, A. V., Ferré, S., Le Grand, D., Paoli, A., Gay, E., Poumarat, F. 2014. Overall decrease in the susceptibility of *Mycoplasma bovis* to antimicrobials over the past 30 years in France. *PLoS One 9*, e87672.

Gerchman, I., Levisohn, S., Mikula, I., Lysnyansky, I. 2009. In vitro antimicrobial susceptibility of *Mycoplasma bovis* isolated in Israel from local and imported cattle. *Vet Microbiol 137,* 268-275.

Gudmundsson, A., Erlendsdottir, H., Gottfredsson, M., Gudmundsson, S. 1991. Impact of pH and cationic supplementation on in vitro postantibiotic effect. *Antimicrob Agents Chemother 35,* 2617-2624.

Hannan, P.C.T. 2000. Guidelines and recommendations for antimicrobial minimum inhibitory concentration (MIC) testing against veterinary *Mycoplasma* species. *Vet Res 31,* 373-395.

Heuvelink, A., Reugebrink, C., Mars, J. 2016. Antimicrobial susceptibility of *Mycoplasma bovis* isolates from veal calves and dairy cattle in the Netherlands. *Vet Microbiol 189*, 1-7.

Jelinski, M., Kinnear, A., Gesy, K., Andrés-Lasheras, S., Zaheer, R., Weese, S., McAllister, T.A. 2020. Antimicrobial sensitivity testing of *Mycoplasma bovis* isolates derived from western Canadian feedlot cattle. *Microorganisms 8*, 124.

Klein, U., de Jong, A., Moyaert, H., El Garch, F., Leon, R., Richard-Mazet, A., Rose, M., Maes, D., Pridmore, A., Thomson, J.R., Ayling R.D. 2017. Antimicrobial susceptibility monitoring of *Mycoplasma hyopneumoniae* and *Mycoplasma bovis* isolated in Europe. *Vet Microbiol 204*, 188-193.

Klein, U., de Jong, A., Youala, M., El Garch, F., Stevenin, C., Moyaert, H., Rose, M., Catania, S., Gyuranecz, M., Pridmore, A., Ayling, R.D. 2019. New antimicrobial susceptibility data from monitoring of *Mycoplasma bovis* isolated in Europe. *Vet Microbiol 238*, 108432.

Kronvall, G. 2010. Normalized resistance interpretation as a tool for establishing epidemiological MIC susceptibility breakpoints. *J Clin Microbiol 48*, 4445-4452.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol 35*, 1547-1549.

Lerner, U., Amram, E., Ayling, R.D., Mikula, I., Gerchman, I., Harrus, S., Teff, D., Yogev, D., Lysnyansky, I. 2014. Acquired resistance to the 16-membered macrolides tylosin and tilmicosin by *Mycoplasma bovis*. *Vet Microbiol 168*, 365-371.

Liu, Y., Xu, S., Li, M., Zhou, M., Huo, W., Gao, J., Liu, G., Kastelic, J.P., Han, B. 2020. Molecular characteristics and antibiotic susceptibility profiles of *Mycoplasma bovis* associated with mastitis on dairy farms in China. *Prev Vet Med 182*, 105106.

Maunsell, F.P., Donovan, G.A. 2009. *Mycoplasma bovis* Infections in Young Calves. *Vet Clin North Am Food Anim Pract 25*, 139-177.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle. *J Vet Intern Med 25*, 772-783.

Parker, Alysia M., Shukla, A., House, J.K., Hazelton, M.S., Bosward, K.L., Kokotovic, B., Sheehy, P.A. 2016. Genetic characterization of Australian *Mycoplasma bovis* isolates through whole genome sequencing analysis. *Vet Microbiol 196*, 118-125.

Rosenbusch, R.F., Kinyon, J.M., Apley, M., Funk, N.D., Smith, S., Hoffman, L.J. 2005. In vitro antimicrobial inhibition profiles of *Mycoplasma bovis* isolates recovered from various regions of the United States from 2002 to 2003. *J Vet Diagn Invest 17*, 436-441.

Stipkovits, L., Ripley, P.H., Tenk, M., Glávits, R., Molnár, T., Fodor, L. 2005. The efficacy of valnemulin (Econor®) in the control of disease caused by experimental infection of calves with *Mycoplasma bovis. Res Vet Sci 78*, 207-215.

Sulyok, K.M., Kreizinger, Z., Fekete, L., Jánosi, S., Schweitzer, N., Turcsányi, I., Makrai, L., Erdélyi, K., Gyuranecz, M. 2014. Phylogeny of *Mycoplasma bovis* isolates from Hungary based on multi locus sequence typing and multiple-locus variable-number tandem repeat analysis. *BMC Vet Res 10*, 108.

Sulyok, K.M., Kreizinger, Z., Wehmann, E., Lysnyansky, I., Bányai, K., Marton, S., Jerzsele, A., Rónai, Z., Turcsányi, I., Makrai, L., Jánosi, S., Nagy, S.Á., Gyuranecz, M. 2017. Mutations associated with decreased susceptibility to seven antimicrobial families in field and laboratory-derived *Mycoplasma bovis* strains. *Antimicrob Agents Chemother 61*, e01983-16.

Ter Laak, E.A., Noordergraaf, J.H., Verschure, M.H. 1993. Susceptibilities of *Mycoplasma bovis*, *Mycoplasma dispar,* and *Ureaplasma diversum* strains to antimicrobial agents in vitro. *Antimicrob Agents Chemother 37*, 317-321.

Thomas, A., Nicolas, C., Dizier, I., Mainil, J., Linden, A. 2003. Antibiotic susceptibilities of recent isolates of *Mycoplasma bovis* in Belgium. *Vet Rec 153*, 428-431.

Toutain, P.L., Bousquet-Mélou, A., Damborg, P., Ferran, A.A., Mevius, D., Pelligand, L., Veldman, K.T., Lees, P. 2017. En Route towards European Clinical breakpoints for veterinary antimicrobial susceptibility testing: A position paper explaining the VetCAST approach. *Front Microbiol 8*, 2344.

Turnidge, J., Kahlmeter, G., Kronvall, G. 2006. Statistical characterisation of bacterial wild-type MIC

value distributions and the determination of epidemiological cut-off values. *Clin Microbiol Infect 12,* 418-425.

Turnidge, J., Paterson, D.L. 2007. Setting and revising antibacterial susceptibility breakpoints. *Clin Microbiol Rev 20*, 391-408.

Vereecke, N., Bokma, J., Haesebrouck, F., Nauwynck, H., Boyen, F., Pardon B., Sebastiaan, T. 2020. High quality genome assemblies of *Mycoplasma bovis* using a taxon-specific Bonito basecaller for MinION and Flongle long-read nanopore sequencing. *BMC Bioinformatics 21*, 517

Yair, Y., Borovok, I., Mikula, I., Falk, R., Fox, L.K., Gophna, U., Lysnyansky, I. 2020. Genomics-based epidemiology of bovine *Mycoplasma bovis* strains in Israel. *BMC Genomics 21*, 70.

GENOME-WIDE ASSOCIATION STUDY REVEALS GENETIC MARKERS FOR ANTIMICROBIAL RESISTANCE IN *MYCOPLASMA BOVIS*

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ABSTRACT

Mycoplasma bovis causes many health and welfare problems in cattle. Due to the absence of clear insights in transmission dynamics and the lack of a registered vaccine in Europe, control mainly depends on antimicrobial therapy. Unfortunately, antimicrobial susceptibility testing is usually not performed, because it is time consuming and no standard protocol or clinical breakpoints are available. Fast identification of genetic markers associated with acquired resistance may at least partly resolve former issues. Therefore, the aim of this study was to implement a first genome-wide association study (GWAS) approach to identify genetic markers linked to antimicrobial resistance in *M. bovis* using rapid long-read sequencing. High quality genomes of hundred *M. bovis* isolates were generated by nanopore sequencing and categorized into wild type and non-wild type isolates. Subsequently, a k-mer based GWAS was performed to link genotypes with phenotypes based on different epidemiological cutoff (ECOFF) thresholds. This resulted in potential genetic markers for macrolides (gamithromycin, tylosin; A2058G$^{+/+}$ in the 23S rRNA gene and Gln93His in the L22 protein of the 50S ribosomal unit) and enrofloxacin (Ser83Phe or Glu87Gly/Val in gyrA and Ser80Ile, Ser81Pro or Asp84Asn/Tyr/Val/Gly in parC). Also, for tilmicosin and the tetracyclines, previously described mutations in both 23S rRNA alleles (G748A$^{+/+}$) and one or both 16S rRNA alleles (A965T, A967T, T1199C, C1992A) were observed. In addition to two new 16S rRNA mutations (A1408G$^{+/-}$ and G1488A$^{+/-}$) associated with gentamicin resistance. In conclusion, this study shows the potential of quick high quality nanopore whole genome sequencing and GWAS in the evaluation of phenotypic ECOFF thresholds and the rapid identification of *M. bovis* strains with acquired resistance.

Key words: epidemiological cutoff, fluoroquinolones, gamithromycin, gentamicin, macrolides, nanopore sequencing

INTRODUCTION

Mycoplasma bovis (*M. bovis*) is an important veterinary pathogen causing various diseases in cattle, such as pneumonia, mastitis and arthritis (Maunsell and Donovan, 2009; Maunsell et al., 2011). Transmission pathways and pathophysiology are not fully understood, hampering development of effective prevention and control (Calcutt et al., 2018; Parker et al., 2018). Also, no effective, commercial vaccine is available. Therefore, the most important way to control an outbreak of *M. bovis* associated diseases remains the adequate use of antimicrobials (Calcutt et al., 2018). *M. bovis* is naturally resistant against beta-lactam antibiotics and (potentiated) sulphonamides (Gautier-Bouchardon, 2018). Given that the use of critically important fluoroquinolones as first intention treatment in animals is strongly discouraged (Aidara-Kane et al., 2018), empiric therapy is mainly limited to florfenicol, tetracyclines and macrolides (Gautier-Bouchardon et al., 2014; Lysnyansky and Ayling, 2016).

Worldwide, an overall increase in acquired resistance *in M. bovis* for mostly macrolides and tetracyclines, but also for florfenicol, lincosamides and fluoroquinolones is reported (Gautier-Bouchardon et al., 2014; Cai et al., 2019; Klein et al., 2019; Bokma et al., 2020a; García-Galán et al., 2020; Liu et al., 2020). To rationalize antimicrobial use for *M. bovis*, there is an urgent need for a rapid and meaningful antibiogram. Unfortunately, phenotypical AST of *M. bovis* is time consuming (up to two weeks), difficult to compare between studies as no standard protocol is available, and almost impossible to translate into *in vivo* results, considering the absence of *M. bovis*-specific clinical breakpoints (CBPs). Therefore, phenotypical AST is not routinely used in practice.

A genetic approach may at least partly resolve former issues, since it is faster and more standardized for AST in *M. bovis* (Ellington et al., 2017; Kinnear et al., 2020). Molecular detection of antimicrobial resistance determinants with methods based on targeted PCR were explored in the past for *M. bovis* (Kong et al., 2016; Sulyok et al., 2018), and targeted gene sequencing for macrolide resistance has already been implemented in research and development settings for human *Mycoplasma pneumoniae* community-acquired infections (Pereyre et al., 2016). Recently, the association between point mutations identified with whole genome sequencing and phenotypic antimicrobial resistance (AMR) have been explored in specific regions of three *M. bovis* strains (Ledger et al., 2020) and on a large set of isolates for macrolides (Kinnear et al., 2020). However, these targeted approaches may result in a narrowed view and potential new genomic alterations within genes, operons, or even promotor, enhancer and/or inhibitory regions might be overlooked (Jaillard et al., 2018;

Kinnear et al., 2020). One way to overcome this shortcoming, is the use of a genome-wide association study (GWAS) to reveal both previously described and novel associations between genotype and phenotype (Coll et al., 2018). In addition, this approach can shed new lights to investigate other resistance mechanisms (*e.g.* methylation and transcription) if a whole genome versus phenotype association remains inconclusive. Key to this kind of analysis, is the generation of complete and highly accurate bacterial genomes. While short-read sequencing approaches have been typically considered as gold standard for sequence accuracy, they result in highly contiguous genome assemblies for *M. bovis* due to its distinct genomic architecture. This is mainly due to a low GC content (29.3%), many highly repetitive regions and the use of a distinct genetic code (Translation table 4) (Razin et al., 1998). Nowadays, high quality (complete and accurate) long-read sequencing approaches have shown to be promising for all-in-one diagnostic workflows (including identification, strain typing, and possibly AMR detection), enormously reducing costs and turnaround time (Bokma et al., 2020c; Vereecke et al., 2020). The aim of this study was to identify known and potential new genetic markers linked with AMR phenotypes in a collection of 100 *M. bovis* isolates, exploiting the power of a genome-wide association approach on high quality and complete nanopore sequenced *M. bovis* genomes.

MATERIALS AND METHODS

Mycoplasma bovis collection and identification

One hundred *M. bovis* isolates obtained from Belgian cattle between 2014-2019 were collected and described in a previous study (Bokma et al., 2020a). Briefly, the *M. bovis* strains were isolated from diagnostic samples (nasal and ear swabs, bronchoalveolar lavage fluid, milk, joint fluid and abdominal fluid). All samples were cultured on a selective-indicative agar plate as described before (Bokma et al., 2020b), and identified with MALDI-TOF MS (Bokma et al., 2019). The isolates were stored at -80°C until phenotypic AST, then aliquots were stored at -20°C until nanopore sequencing was performed on freshly grown cultures.

Phenotypic antimicrobial susceptibility testing and interpretation

The EUCAST Subcommittee recommends the epidemiological cutoff value (ECOFF) as the primary comparator for identifying an association between genotype from whole genome sequence data and phenotype (Ellington et al., 2017). The ECOFF distinguishes *M. bovis* isolates in wild-type (WT) bacterial populations and those with acquired resistance (non-wild

type; non-WT) based on the minimum inhibitory concentration (MIC). In a previous study, MICs of *M. bovis* isolates and *M. bovis* PG45 were obtained with microbroth dilution for tetracyclines (oxytetracycline; OXY, doxycycline; DOXY), macrolides (tilmicosin; TIL, tylosin; TYL, and gamithromycin; GAM), florfenicol (FLOR), gentamicin (GEN), enrofloxacin (ENRO) and tiamulin (TIA), using custom-made 96-U-bottom well Sensititre microplates (Thermofisher), resulting in different ECOFF values depending on the method used (Bokma et al., 2020a). In the present study, these different ECOFF values obtained by the visual estimation and two statistical methods (Normalized Resistance Interpretation (NRI) and the Iterative Statistical Method (95/99%)) were explored to determine the best ECOFF for the GWAS on *M. bovis*. As no ECOFF could be determined for TIL, isolates with MIC ≥ 32 µg/mL were categorized as non-WT population, as previously suggested by Lerner et al. (2014).

Generation of high quality and complete *M. bovis* genome assemblies with nanopore sequencing

Total DNA of 100 *M. bovis* recent field isolates was extracted and subjected to whole-genome long-read nanopore sequencing as described previously (Bokma et al., 2020c). Sequencing was performed using native DNA sequencing with the Rapid Barcoding Sequencing Kit (SQK-RBK004; Oxford Nanopore Technologies (ONT)). A total of 12 strains were sequenced per run on an R9.4.1 flow cell (ONT) using a MinION device. In each sequencing run, the *M. bovis* PG45 type strain (ATCC®25523™) was included as a positive quality control and mock inoculated broth was used as negative control. Raw data (fast5 files) were collected in the MinKnow software (v.3.6.5; ONT) and used in downstream bioinformatics analyses. *M. bovis* genomes were assembled as described before. Raw read files were basecalled using a *M. bovis*-specific trained Bonito basecalling model on Bonito (v.0.2.2; ONT) to generate high quality and reliable *M. bovis* sequences (Vereecke et al., 2020). Resulting reads were assembled into genomes using canu (v.1.9; Koren et al., 2016) and Medaka (v.1.0.0; ONT). Final genome assemblies were annotated using the Prokka rapid prokaryotic genome annotation pipeline (v.1.14.6; Seemann, 2014) and absence of plasmids was verified by contig evaluation and abricate (v.1.0.1; https://github.com/tseemann/abricate; Carattoli et al., 2014).

Genome-wide association study

Firstly, we assessed the quality of all *M. bovis* genomes to ensure only high quality and complete genomes were included in downstream GWAS. Only genomes with sufficient median depth (>30X) and genome completeness (at least 224/226 marker genes; >99%) were used. Genome quality control was done using QUAST (v.5.0.2; Gurevich et al., 2013) and CheckM (v.1.1.0; Parks et al., 2015) using the *Mycoplasma* spp. (n = 226 markers from 83 genomes) gene marker set. When all gene markers were present, a genome was considered 100% complete. Contamination was assessed using Kraken2 classification (v2.0.9-beta; Wood et al., 2019) of contigs against the k2_pluspf_20200919 database. Contaminating contigs were removed along with duplicated contigs based on their size and effect on completeness.

A k-mer based GWAS was performed to link phenotypes to genotypes. To this end the DBGWAS software (v.0.5.4; Jaillard et al., 2018) was used at default settings. The DBGWAS algorithm relies on extended k-mer searches based on compacted De Bruijn graphs to associate genetic variants with clear phenotypes. Firstly, a list was generated to link genotypes to phenotypes by categorizing the genomes as WT (designated 0), non-WT (designated 1) or undefined (designated as NA) if no phenotypic data was available. This was done for each antimicrobial drug tested and using the four ECOFF methods as mentioned above. This was given as input along with a phylogenetic tree of all genomes, generated through CSI phylogeny using the *M. bovis* PG45 type strain genome (NC_014760) as reference. Final DBGWAS visualization output was evaluated by significance (*p*- and q-values), annotation, and allele frequencies of each phenotypic category. Final DBGWAS analysis output links are available upon request. Subsequently, designated “suspicious” gene targets were extracted from the annotated genomes, aligned using mafft (v.4.471; Katoh and Standley, 2013), manually curated, and analyzed for non-synonymous protein or nucleotide mutations in protein-coding sequence or ribosomal RNAs (rRNAs), respectively. A similar strategy was used for previously published genetic markers if insufficient phenotypic data (tilmicosin, tetracyclines and gentamicin) were available. Results were visualized using Interactive Tree of Life (iTOL) (v.5.7; Letunic and Bork, 2019).

RESULTS

Phenotypic AST and the evaluation of high-quality *M. bovis* genomes

Results of the phenotypic AST resulting in the determination of the ECOFFs were published elsewhere (Bokma et al., 2020a). All *M. bovis* PG45 tests showed comparable MIC values, classifying them to the WT population for each antimicrobial tested. Only high quality and complete (n=95 of 100) genomes were included in the GWAS, therefore a total of 5 genomes were excluded from subsequent GWAS analyses. Classification of *M. bovis* strains into (non-) WT or susceptible/resistant isolates was based on the ECOFFs as previously determined by Bokma and colleagues (2020a). Results for the 95 isolates and *M. bovis* PG45 in this study are shown in Table 1.

Table 1. Distribution of antimicrobial susceptibility (WT: wild-type; n-WT: non wild-type) of 95 nanopore sequenced *M. bovis* isolates and *M. bovis* PG45 per epidemiological cut-off (ECOFF) method (Visual estimation, Normalized Resistance Interpretation (NRI), and Iterative Statistical Method; ISM). Determination of ECOFFs were previously published (Bokma et al., 2020a).

	Visual estimation			**NRI**			**ISM (95/99%)**		
Antimicrobial	EC	WT	n-WT	EC	WT	n-WT	EC	WT	n-WT
Florfenicol	>16	91	4	>16	91	4	>8/16 (+)	89/91	6/4
Oxytetracycline	>8	94	0	>8	94	0	>4/8 (+)	92/94	2/0
Doxycycline	>4	94	0	>2	91	3	>1/2 (+)	82/91	12/3
Tilmicosin	ND	-	-	>1024	-	-	ND	-	-
Tylosin	>32	46	50	>128 #	46	50	ND	-	-
Gamithromycin	>64	53	43	>128 #	56	40	ND	-	-
Gentamicin	>16	95	1	> 8	94	2	>4/4 (-)	91/91	5/5
Tiamulin	>0.5	95	1	>0.125	80	13	>0.06/0.06 (-)	59/59	34
Enrofloxacin	>2	85	8	> 1	83	10	>1/2 (±)	83/85	10/8

= tentative estimate, as standard deviation >1.2 $\log_2$. * plots for residuals were checked and categorized in either well fit (+), poor fit (±) or no fit (−) corresponding to whether the subset values are reliable or not. ND: not possible to determine. NA: not available

GWAS analysis based on different ECOFF methods

First, the GWAS analysis was applied to the different distributions of WT and non-WT based on ECOFFs determined by visual estimation, NRI, and ISM95/99% approaches (Table 1). The most significant (*p* and *q* values) results for ENRO and TYL were seen when ECOFF was based on the visual estimation method. For GAM a negligible difference between the visual estimation and NRI was observed (Supplement 1). Unfortunately, for FLOR, OXY, DOXY, TIL, GEN and TIA, the GWAS analysis was not successful as either (i) no, (ii) too little (n<5) strains were addressed to the (non-)WT group or (iii) no clear association could be made between the classified genotypes and observed phenotypes. As not all methods could be applied to all macrolides, and results of the visual estimation were for the most part more

significant than the other methods, from here on GWAS results are shown based on ECOFFs determined by the visual estimation method as described in Bokma et al. (2020a).

Mutations in the *M. bovis gyrA* and *parC* encoding genes are associated with enrofloxacin resistance

A successful GWAS analysis for fluoroquinolone ENRO could be performed as the non-WT population contained 8 isolates out of 96 (95 field isolates + PG45) total high-quality genomes. Two significant components were identified covering the *gyrA* (Fig. 1A) and *parC* (Fig. 1B) gene targets for the ENRO phenotype. These genes encode for the DNA gyrase subunit A and DNA topoisomerase 4 subunit A protein, respectively. Other components were analysed but did not show an association with the ENRO resistant phenotype. In-depth analysis of these "suspected" target genes from each genome showed the existence of two non-synonymous mutations (Ser83Phe or Glu87Gly/Val) located in the Quinolone Resistant Determining Region (QRDR). In addition, four possible mutations (Asp79Asn, Ser80Ile, Ser81Pro and Asp84Asn/Tyr/Val/Gly) were identified in the ParC protein (Fig. 2; orange). The MIC values for the *M. bovis* isolates and their associated mutations in GyrA and ParC are shown in more detail in Fig. 3.

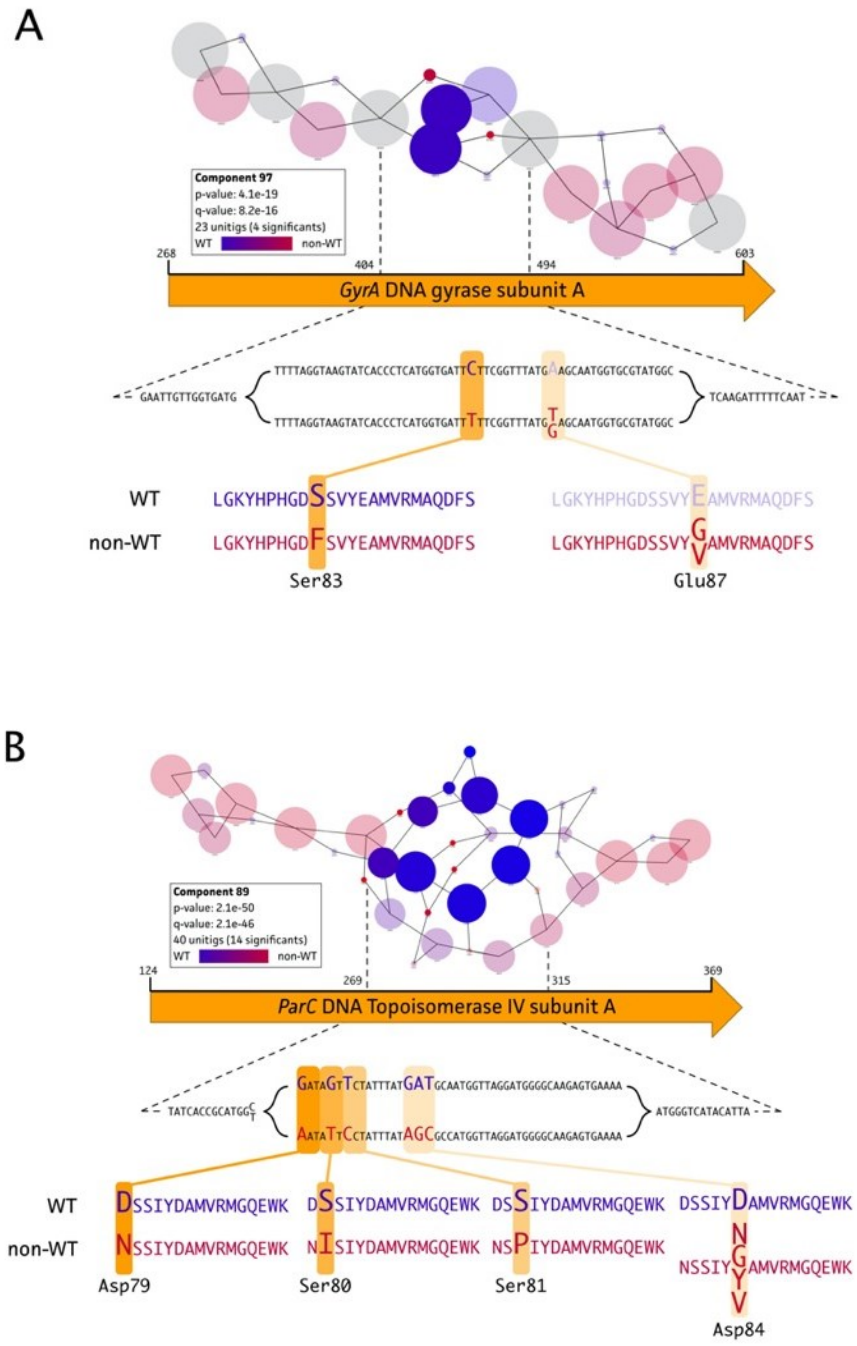

Figure 1. DBGWAS analysis for enrofloxacin resistance in 95 Belgian *M. bovis* isolates and *M. bovis* PG45. Two significant associations between the enrofloxacin non-wild type (non-WT) phenotype (n=8) and genotype could be found for two known fluoroquinolone gene targets, including the *gyrA* (a) and *parC* (b) genes. Further in-depth analysis showed the identification of 2 and 4 non-synonymous mutations in the GyrA and ParC protein, respectively. Amino acid positions are labelled according to classical *E. coli* numbering.

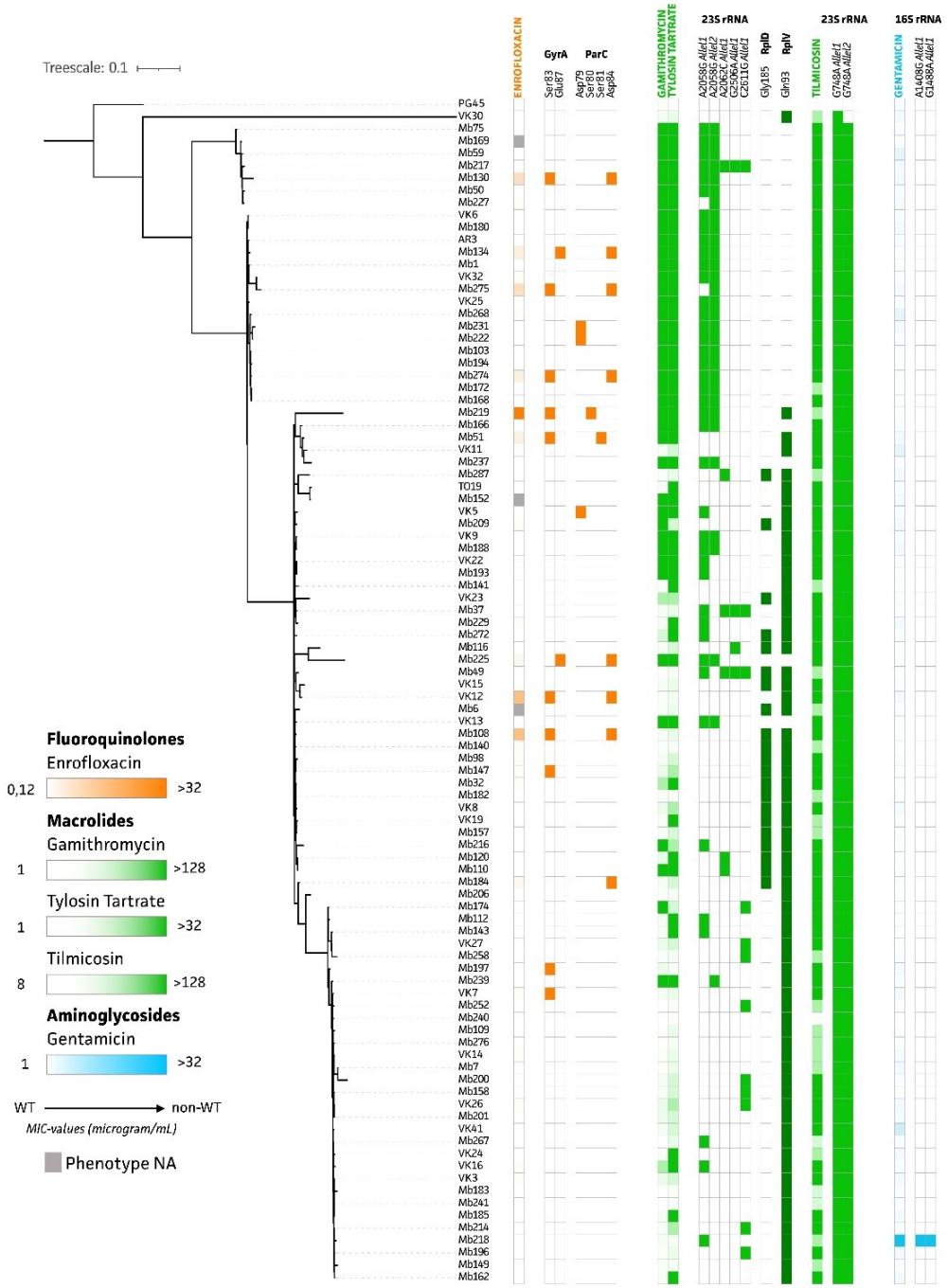

Figure 2. Distribution of phylogenetic tree, MIC values and (non-synonymous) mutations of 95 Belgian *M. bovis* field isolates and *M. bovis* PG45. Colour lapse shows gradient of MIC values for enrofloxacin (orange), macrolides (gamithromycin, tylosin, tilmicosin; green) and gentamicin (blue), while coloured blocks show the presence/absence of (non-synonymous) mutations. Nucleotide and amino acid positions are labelled according to classical *E. coli* numbering.

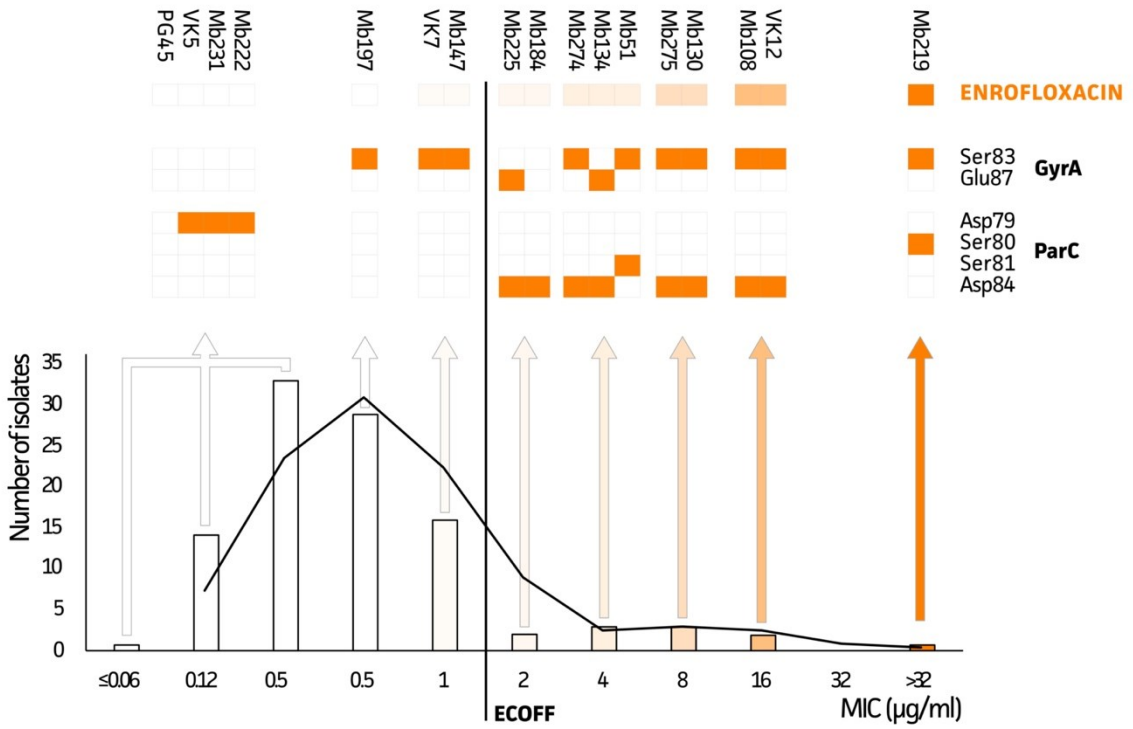

Figure 3. Distribution of MIC values for 100 Belgian *M. bovis* isolates and *M. bovis* PG45 and their associated mutations in gyrA and parC. All strains, except Mb184, with a double mutation in gyrA and parC show MIC values above the ECOFF (n=10).

Macrolide resistance in *M. bovis* is associated with genetic markers in the 23S ribosomal subunit

TIL had only one isolate belonging to the WT (PG45) population, therefore a GWAS could not be performed. However, the determined ECOFF allowed us to identify a known mutation by investigating previously reported resistance target genes. The G748A mutation in domain II of both 23S rRNA alleles could be linked to the non-WT population (n = 95) and was not present in PG45 (Fig. 2; green). For the two macrolides, GAM and TYL, 52 and 45 out of 96 isolates (95 field isolates + PG45) belonged to the non-WT population. The DBGWAS analysis highlighted the association of both 23S rRNA gene and the ribosomal operon in the resistance phenotypes of both TYL and GAM. Hence, both 23S rRNA alleles and all 50S accessory ribosomal proteins were extracted and screened for mutations in association to TYL and GAM.

As shown in Figure 5A, comparable significant associations of point mutations at position A2058 and A2062 within domain V of the 23S rRNA gene were shown. The A2058 mutation was found in both alleles, whereas the A2062 mutation was only found in one allele of the 23S rRNA gene (Fig. 5B). 23S rRNA mutations C2062A, G2506A, and C2611G were present in allele 1 of 6, 4, and 12 isolates, respectively, but it was not possible to link them with observed macrolide phenotypes. In addition, both TYL and GAM phenotypes were associated with an operon as suggested by the thread-like structure in the DBGWAS analyses output (Fig. 5C) (Jaillard et al., 2018). DBGWAS k-mer annotation and further analyses of the resulting operon, suggested the association of the ribosomal operon with the GAM and TYL phenotype (Fig. 5D). Further analyses of the ribosomal genes revealed GAM/TYL resistance-associated mutations in the *rplD* and *rplV* gene, encoding for 50S ribosomal protein 4 (L4) and 22 (L22). While no clear association of the non-synonymous Gly185Val/Arg mutation in the L4 protein with the n-WT of TYL/GAM was identified, the Gln93His mutation in L22 suggested an association with (combined) GAM and TYL non-WT type phenotypes. The latter was observed in all isolates (n=35) harboring the transition at the A2058 position in domain V of one or both alleles of the 23S rRNA, of which 27 strains lacked the Gln93His mutation in the L22 ribosomal protein. Still 6 out of 41 double TYL and GAM resistant isolates showed distinct mutation patterns which could not be linked to a specific resistance phenotype (Fig. 2; green), and 15% (6/41) of isolates showing a double TYL and GAM resistance phenotype could not be linked to a specific non-WT phenotype (Fig. 2; green). This was also the case for 7 and 13 strains which belonged to only one of GAM or TYL non-WT populations, respectively.

Mutations in the 16S rRNA possibly associated with tetracycline resistance

Depending on the ECOFF used, no to limited (13%) phenotypic tetracycline resistance was observed in this study. Nevertheless, previously reported 16S rRNA mutations possibly associated with resistance were observed. In all isolates one or more mutatations were identified, except for *M. bovis* PG45 (MIC ≤ 0.12 µg/mL), where no mutations was observed on residue 965, 967, 1058, 1192 or 1199 of the 16S rRNA. In all 95 field isolates A965T and A967T mutations are present, with additional mutations C1192A (8 in one allel, 23 in both alleles) and T1199C (26 in one allel, 3 in both alleles). No clear association with increased MIC values was observed, although an increase in number of mutations resulted in increased MIC values (Fig. 4).

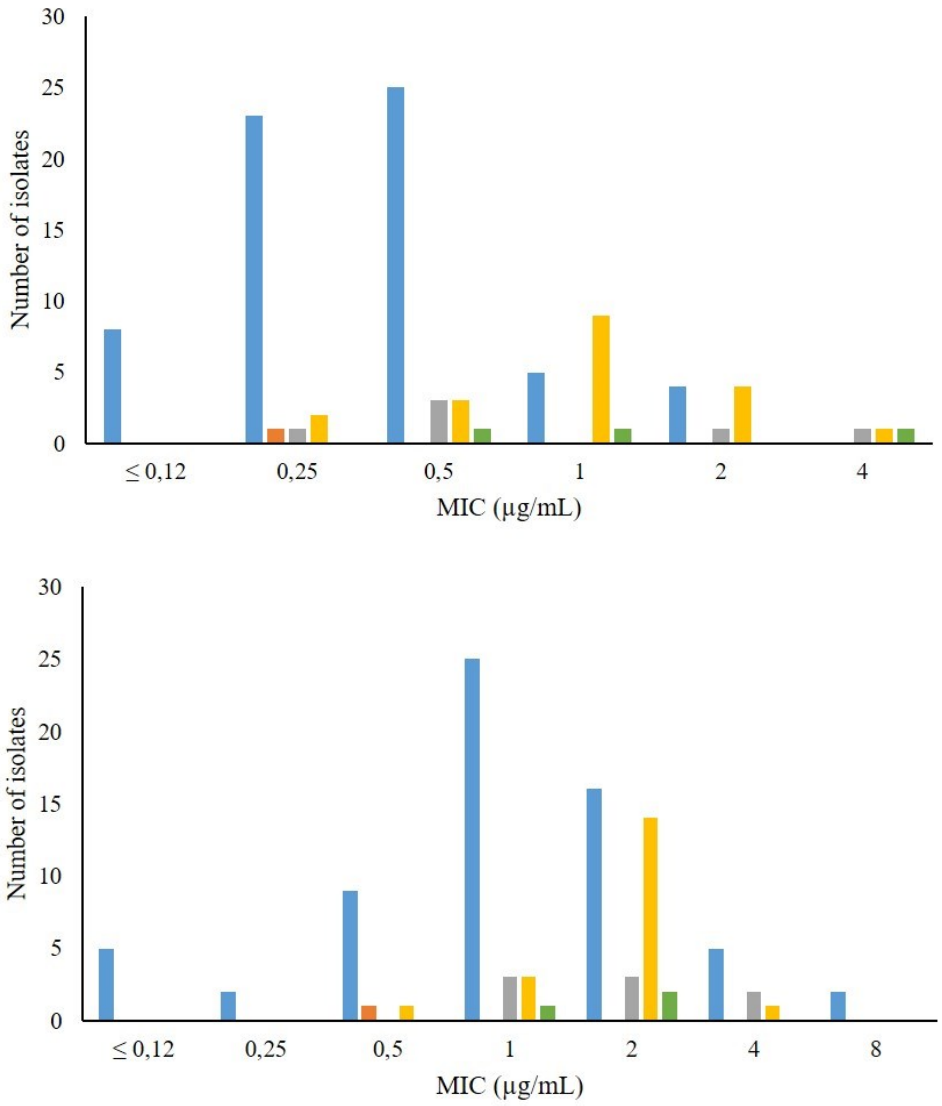

Figure 4. Distribution of MIC values for 95 *M. bovis* isolates and *M. bovis* PG45 and the number of mutations at position 1192 and 1199 in the 16S rRNA (0: blue, 1: orange, 2: grey, 3: yellow, 4: green) for doxycycline (above) and oxytetracycline (down).

Marker mutation in 16S rRNA observed in gentamicin resistant *M. bovis* isolate

For GEN the GWAS could not be performed. Nevertheless, one of the *M. bovis* isolates (Mb218) showed a significantly higher MIC (64 µg/mL) as compared to the WT population (< 32 µg/mL). Since GEN is known to act on the 16S ribosomal subunit, all small ribosomal proteins and both 16S rRNA alleles were manually checked for mutations. Two transversions (A1408G and G1488A) in either one of both 16S rRNA alleles were observed in the Mb218 strain. Since none of the WT isolates harbored these mutations and both A1408 and G1488 transversions were located at or near the GEN binding site of domain II of the 16S rRNA, these mutations are possibly marker mutations for GEN resistance in *M. bovis* (Fig. 2, blue).

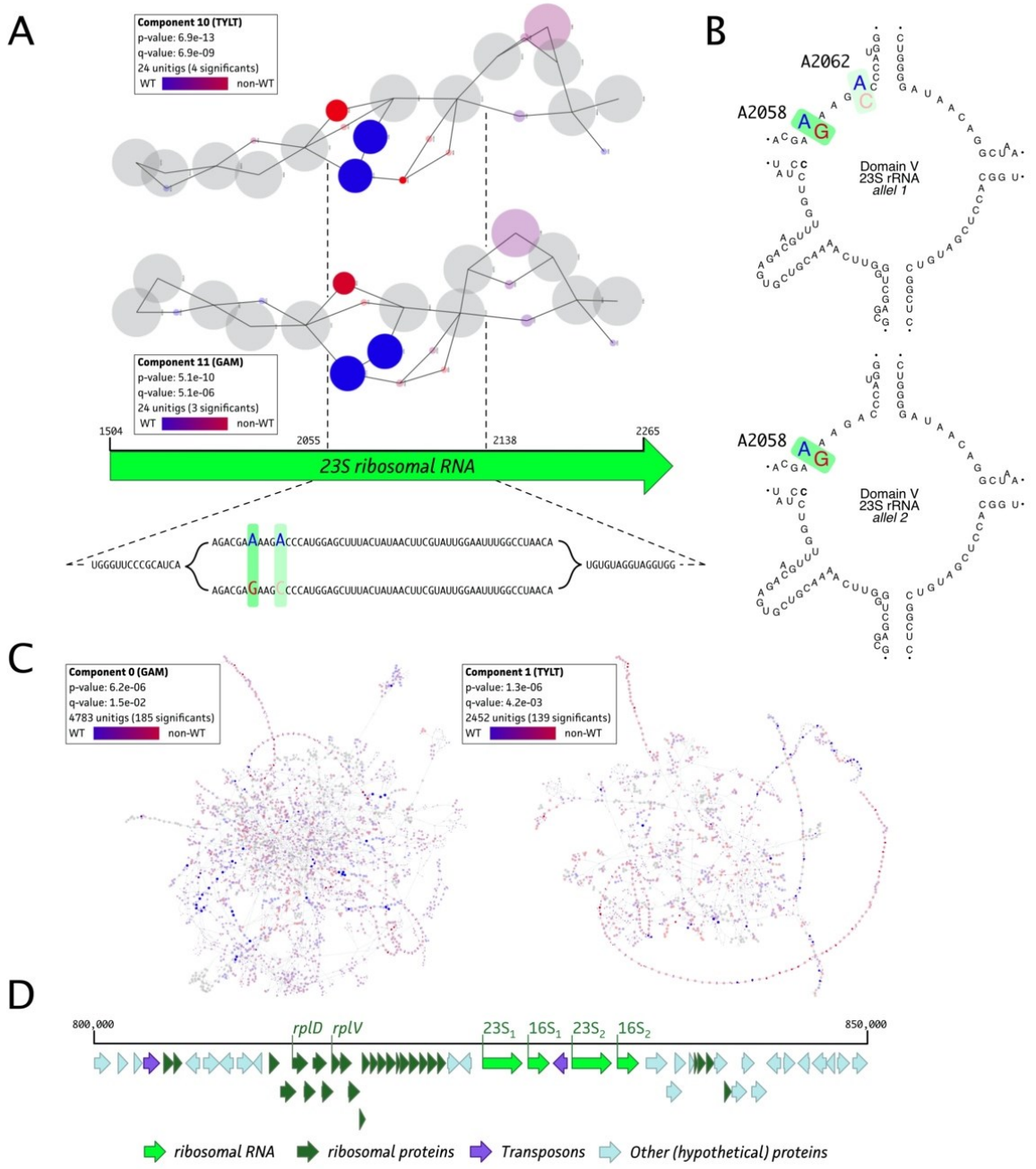

Figure 5. DBGWAS analysis for gamithromycin (GAM) and tylosin (TYL) resistance in 95 Belgian *M. bovis* isolates and *M. bovis* PG45. (A) Association of GAM (non-WT=43) and TYL (non-WT=50) genotypes with phenotypes, resulted in a shared 23S rRNA target association. (B) Secondary structure of Domain V of both 23S rRNA alleles, showing the observed mutations (A2058 and A2062). 23S rRNA positions are labelled according to classical *E. coli* numbering. (C) DBGWAS analysis output highlights a complex k-mer web, including continuous k-mer strands, suggesting the association of a genetic operon with the phenotype. (D) Genetic context of the *M. bovis* ribosomal operon, indicating known GAM and TYL drug target genes (23S rRNA, *rplD*, and *rplV*).

DISCUSSION

In this study, we exploited a GWAS approach to associate the *M. bovis* genotype to phenotypic antimicrobial susceptibility test results. High quality complete and accurate whole genomes were generated using nanopore sequencing and an optimized taxon-specific base calling model and assembly as previously described (Vereecke et al., 2020). In addition, different methods to determine ECOFFs and therefore the delineation of the *M. bovis* WT population and strains with acquired resistance (non-WT) were explored. The GWAS analysis showed significant and clear results for the critically important antibiotics ENRO, GAM, and TYL, as enough strains were available belonging to the WT or non-WT populations. These antimicrobials require top concern, according to the World Health Organization and the World Organization for Animal Health (WHO, 2017; OIE, 2019).

In this study we identified several previously reported mutations for ENRO resistance in *M. bovis* (gyrA: Ser83Phe, Glu87Gly/Val; parC: Ser80Ile, Ser81Pro, and Asp84Asn/Tyr/Gly/Val), supporting the relevance of the obtained output. In addition, a new genetic marker in parC (Asp79Asn) was identified and associated with acquired ENRO resistance. This mutation was previously described in clinical *M. synoviae* isolates and *in vitro* mutated *M. agalactiae* isolates (Lysnyansky et al., 2013; Tatay-Dualde et al., 2017). No mutations in the GyrB protein could be associated with the phenotypes (data not shown), which was expected because such mutations are described to be associated with evolutionary mutations for which we corrected by implementing the phylogenetic tree (Lysnyansky et al., 2009; García-Galán et al., 2020). Any single gyrA mutation (Ser83Phe or Glu87Gly/Val) was observed in strains with MIC-value of 0.5 and 1 µg/ml, as was previously described for Ser83Phe in Israeli *M. bovis* isolates (Lysnyansky et al., 2009). Even though these strains still belonged to the WT population according to an ECOFF of >1 µg/mL, the isolates were all on the right-hand side of the normal distribution and therefore close to the ECOFF. In Israeli isolates, an additional mutation in Asp84Asn (parC) was necessary to obtain resistance (MIC >2 µg/ml) (Lysnyansky et al., 2009), which supports our findings. Mutations in Glu87 (gyrA) were previously only demonstrated after *in vitro* selection and were believed to have no impact on resistance (Khalil et al., 2015; Sulyok et al., 2017). However, in our study the Glu87Gly/Val mutation was associated with elevated MIC values (Mb225: 2 µg/ml and Mb134: 4 µg/ml) when co-occurring with a *parC* gene (Aps84) mutation. It was previously described that a mutation on the same position (Asp84Asn) results in a 2-fold increase of the MIC-value (Lysnyansky et al., 2009; Khalil et al., 2015; Sulyok et al., 2017), which possibly

explains the increased MIC-value of the Glu87 mutation. Influence on MIC values of other mutations on this location (Asp84Tyr/Gly) have not yet been determined (Khalil et al., 2015). Therefore, further research is necessary whether mutations on this location could result in resistance on its own. Hata and coworkers (2019) concluded that single mutations in *parC* do not result in lower susceptibility. However, in our study one isolate (Mb184) contained a single mutation Asp84 in parC with a non-WT phenotype (MIC = 2 µg/ml). Other resistance mechanisms might have been involved, as efflux pumps were identified in *M. hominis* resulting in resistance for fluoroquinolones (Raherison et al., 2002), which cannot be evaluated with the current approach.

In one isolate, highest MIC-values (≥ 32 µg/ml) were obtained for the combination of Ser83Phe and Ser80Ile, which is in line with mutations identified in French, Japanese and Spanish *M. bovis* isolates (Sato et al., 2013; Khalil et al., 2015; Hata et al., 2019; García-Galán et al., 2020).

The GWAS suggested the visual estimated ECOFF of > 2 µg/ml should be lowered to > 1 µg/ml. However, isolates containing only a mutation in Ser83 were still not identified as non-WT by the phenotypical AST but were close to the ECOFF. Therefore, using molecular methods may be superior to the phenotypical AST as an early warning tool for the emerging of AMR in surveillance programs. In addition, on the individual animal or herd level, it would be recommended to avoid the use of ENRO when at least one mutation is found, even though phenotypical susceptibility testing shows susceptibility, as more selection pressure would result in additional mutations, and therefore increased MIC values.

All non-WT isolates for the 16-membered-ring macrolide TIL contained the G748A mutation in domain II of both 23S rRNA alleles, which was also observed in previous studies (Sato et al., 2017; Hata et al., 2019; Kinnear et al., 2020; Ledger et al., 2020). An additional mutation at position 2058, was associated with GAM (15-membered-ring) and TYL (16-membered-ring) resistance in our GWAS. This combination of mutations was also observed in previous TYL and TIL resistant isolates (Lerner et al., 2014; Kong et al., 2016; Khalil et al., 2017). The mutation at A2058 has also been associated with macrolide and lincosamide resistance in *M. bovis* before, although in clinical Spanish isolates, there was only an association with lincosamide resistance (Sato et al., 2017; García-Galán et al., 2020). In our study the Gln93His mutation in the L22 protein was observed in 73% of isolates. This is in the same range as reported by Lerner et al. (2014) and is below the 100% incidence in Kinnear et al. (2020). The C2611G mutation was identified in our study, but was not associated with increased resistance phenotypes, which is in line with observations in *M. pneumoniae*

(Pereyre et al., 2016). The A2062 and G2506A mutations have been suggested to be linked to florfenicol and pleuromutilin (*e.g*. tiamulin) resistance before in *M. bovis* (Sulyok et al., 2017; Hata et al., 2019). Even though these mutations were identified in some of the currently investigated *M. bovis* isolates, no clear association was identified with the phenotypical resistance against any of the tested antimicrobials. The associations between other mutations and macrolide resistance of *M. bovis* were non-conclusive (data not shown). This could be due to a less black-and-white division between WT and non-WT populations, representing an overlapping grey area, or insufficient distribution of the isolates in this study. Together, this will result in errors in current used methodology. However, it is more likely that we are not fully understanding the genotypic basis of the phenotypic resistance yet (Ellington et al., 2017; Ledger et al., 2020). For example, other resistance mechanisms, such as target-site modification by methylation has been described for macrolides in *Streptomyces fradiae* (Liu and Douthwaite, 2002). Analogous mutations have been identified in *M. bovis* but have not yet been associated with AMR (Ledger et al., 2020). Another macrolide AMR mechanism that was not fully investigated in current research, is the efflux of the drug by ABC-transporters. While (ABC-type) efflux pumps were identified in the *M. bovis* genome (data not shown), their causal link with macrolide resistance still has to be confirmed using targeted mutagenesis, efflux pump inhibitors or gene expression analyses using RNA-sequencing. However, for *M. pneumoniae* it has been shown that efflux pumps (possibly ABC-type) are involved in resistance to macrolides (Li et al., 2017). Cross-resistance with other antimicrobials not included in this study, such as lincosamides, may also be a likely explanation. Cross-resistance between the macrolides and lincosamides is frequently described for *Mycoplasma* species, as both classes of molecules bind to domain V of the 23S rRNA and the L22 ribosomal protein (Sulyok et al., 2017; Prats-van der Ham et al., 2018). Mutation of A2059G in both 23S rRNA alleles was also seen in lincomycin resistant *M. bovis* isolates (Sulyok et al., 2017; Hata et al., 2019) and in *M. pneumoniae* strains, where mutations in L4 and L22 ribosomal proteins were associated with both macrolide and lincosamide resistance (Pereyre et al., 2004).

When insufficient strains belong to the (non-)WT population (*e.g.* OXY, DOXY, FLOR, TIL, GEN, TIA) a GWAS analysis is not successful or renders inconclusive outputs. Hence the genetic profiling of AMR is limited to the detection of previously described mutations available in literature. For OXY and DOXY, several mutations previously associated with tetracycline resistance were observed, although the mutation at site 1058, previously described in France and Japan was not observed in this study (Amram et al., 2015, Khalil et

al., 2017; Hata et al., 2019). For a part of the isolates, the data suggests an increase in MIC value when more alterations are observed, in the remaining part another unknown mechanism (*e.g.* efflux mechanisms) may be involved in increasing the MIC values (Chopra and Roberts, 2001). Alterations at 1192 in one or two alleles were also associated with spectinomycin resistance (Sulyok et al., 2017; Hata et al., 2019), but could not be confirmed in this study as spectinomycin was not included in the phenotypic AST. In the case of GEN, only one strain was classified as non-WT and was the only strain showing mutations (A1408G and G1488A) in either one of both 16S rRNA alleles. Due to their approximate localization to the known gentamicin-binding region of the 16S rRNA, both mutations are suggested to contribute to GEN acquired resistance. Even though RNA methyltransferases were extensively investigated in aminoglycoside resistance, mutations were shown to confer aminoglycoside resistance in *Mycobacterium abcessus* (Nessar et al., 2011; Doi et al., 2016). Whether these mutations are really resulting in higher GEN MIC values and if so, whether both mutations are required or only one is sufficient to show a GEN resistance phenotype, should be addressed in further research.

Investigating the genome by GWAS for nonsynonymous mutations associated with resistance can clarify whether ECOFFs have been rightfully chosen. The present study showed that determination of the ECOFF with the visual estimation method resulted in best agreement between antimicrobial resistant phenotype and genotype for the antimicrobials which had a clear bimodal distribution. Nonetheless, it also showed that statistical methods can be of great help in case of truncated distributions ('tailing'), which are frequently observed for step-by-step resistance mechanisms, such as the fluoroquinolones (ECOFF visual estimation: >2; statistical methods: >1). The determination of ECOFF is very suitable for surveillance and to rapidly recognise development of resistance in a population, as it shows small changes compared to the WT population (Bywater et al., 2006). Although the ECOFF is a good indicator for the determination of acquired resistance, it should be kept in mind that translating ECOFFs to clinical outcomes is discouraged (Bywater et al., 2006). To clinically interpret MIC values and associate these with mutations in the genome, clinical breakpoints for *M. bovis* should be available first. Only then, the concordance between WGS and clinical breakpoints can be assessed.

This study showed nanopore sequencing as rapid new tool to readily determine acquired antimicrobial resistance and support evaluation of ECOFF values in *M. bovis*. Since conventional identification and AST for *Mycoplasma* spp. are highly time-consuming (up to 2 weeks). This approach allows to significantly shorten current sampling-to-result workflow.

Even though here pre-enriched samples were used, implementing nanopore-based approaches immediately on field samples should be a reachable future goal to make identification and AST data of various species readily available. Using GWAS, we were able to reveal genetic markers associated with acquired antimicrobial resistance of *M. bovis* for critically important antibiotics of the fluoroquinolone and macrolide family, were revealed. By using data generated in this kind of analyses, *M. bovis* field strains can be classified as WT or non-WT in a rapid and easy way, which is not possible with current growth-dependent methods and the lack of widely used ECOFFs or CBPs. Therefore, rapid nanopore sequencing may help in antimicrobial decision making, while facing an *M. bovis* outbreak. The applicability can be even more broad by expanding the input of the GWAS analysis with additional phenotypical and genome information of (non-)WT *M. bovis* isolates from different populations.

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CONFLICT OF INTEREST

Sebastiaan Theuns and Hans Nauwynck are co-founders of PathoSense BV, a spin-off company of Ghent University. Nick Vereecke is PhD student at PathoSense BV and Ghent University.

SUPPLEMENTARY DATA

Supplement 1. Results of GWAS on 96 *M. bovis* isolates based on different ECOFF methods

Antimicrobial	Gene	*p*-value	*q*-value	Significance	WT vs nWT
			Visual estimation		
Enrofloxacin	***ParC***	2,07E-50	2,06E-46	40 unitigs (14 significants)	85/8
	GyrA	4,11E-19	8,19E-16	23 unitigs (4 significants)	85/8
Tylosin	**23S rRNA**	6,90E-13	6,87E-09	24 unitigs (4 significants)	46/50
	rOperon	1,28E-06	4,24E-03	2452 unitigs (139 significants)	46/50
Gamithromycin	**23S rRNA**	6,85E-11	6,83E-07	24 unitigs (3 significants)	53/43
	rOperon	4,12E-06	1,37E-02	4929 unitigs (226 significants)	53/43
			Normalized resistance interpretation		
Enrofloxacin	***ParC***	7,38E-43	7,35E-39	40 unitigs (12 significants)	83/10
	GyrA	5,59E-18	1,11E-14	23 unitigs (4 significants)	83/10
Tylosin	**23S rRNA**	6,90E-13	6,87E-09	24 unitigs (4 significants)	46/50
	rOperon	1,28E-06	4,24E-03	2452 unitigs (139 significants)	46/50
Gamithromycin	**23S rRNA**	2,70E-12	2,69E-08	24 unitigs (3 significants)	56/40
	rOperon	6,70E-07	1,33E-03	4833 unitigs (183 significants)	56/40
			ECOFFinder 95%		
Enrofloxacin	***ParC***	7,38E-43	7,35E-39	40 unitigs (12 significants)	83/10
	GyrA	5,59E-18	1,11E-14	23 unitigs (4 significants)	83/10
			ECOFFinder 99%		
Enrofloxacin	***ParC***	2,07E-50	2,06E-46	40 unitigs (14 significants)	85/8
	GyrA	4,11E-19	8,19E-16	23 unitigs (4 significants)	85/8

WT = wild type, nWT = non-wild type

REFERENCES

Aidara-Kane, A., Angulo, F.J., Conly, J., Minato, Y., Silbergeld, E.K., McEwen, S.A., Collignon, P.J., Balkhy, H., Collignon, P., Friedman, C., Hollis, A., Kariuki, S., Kwak, H.S., McEwen, S., Moulin, G., Ngandjio, A., Rollin, B., Rossi, F., Wallinga, D. 2018. World Health Organization (WHO) guidelines on use of medically important antimicrobials in food-producing animals. *Antimicrob Resist Infect Control 17*, 7.

Amram, E., Mikula, I., Schnee, C., Ayling, R.D., Nicholas, R.A.J., Rosales, R.S., Harrus, S., Lysnyanskya, I. 2015. 16S rRNA gene mutations associated with decreased susceptibility to tetracycline in *Mycoplasma bovis*. *Antimicrob Agents Chemother 59,* 796-802.

Bokma, J., Gille, L., De Bleecker, K., Callens, J., Haesebrouck, F., Pardon, B., Boyen, F. 2020a. Antimicrobial susceptibility of *Mycoplasma bovis* isolates from veal, dairy and beef herds. *Antibiotics 9*, 882.

Bokma, J., Pardon, B., Van Driessche, L., Gille, L., Deprez, P., Haesebrouck, F., Boyen, F. 2019. Optimizing identification of *Mycoplasma bovis* by MALDI-TOF MS. *Res Vet Sci 125*, 185-188.

Bokma, J., Van Driessche, L., Deprez, P., Haesebrouck, F., Vahl, M., Weesendorp, E., Deurenberg, R.H., Pardon, B., Boyen, F. 2020b. Rapid identification of *Mycoplasma bovis* from bovine bronchoalveolar lavage fluid with MALDI-TOF MS after enrichment procedure. *J Clin Microbiol 48*, e0004-20.

Bokma, J., Vereecke, N., De Bleecker, K., Callens, J., Ribbens, S., Nauwynck, H., Haesebrouck, F., Theuns, S., Boyen, F., Pardon, B. 2020c. Phylogenomic analysis of *Mycoplasma bovis* from Belgian veal, dairy and beef herds. *Vet Res 51,* 121.

Bywater, R., Silley, P., Simjee, S. 2006. Antimicrobial breakpoints-Definitions and conflicting requirements. *Vet Microbiol 118*, 158-159.

Cai, H.Y., McDowall, R., Parker, L., Kaufman, E.I., Caswell, J.L. 2019. Changes in antimicrobial susceptibility profiles of *Mycoplasma bovis* over time. *Can J Vet Res 83*, 34-41.

Calcutt, M.J., Lysnyansky, I., Sachse, K., Fox, L.K., Nicholas, R.A.J., Ayling, R.D. 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. *Transbound Emerg Dis. 65*, 91-109.

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M.V., Lund, O., Villa, L., Aarestrup, F.M., Hasman, H. 2014. In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother 58*, 3895-3903.

Chopra, I. Roberts, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev 65*, 232-260.

Coll, F., Phelan, J., Hill-Cawthorne, G.A., Nair, M.B., Mallard, K., Ali, S., Abdallah, A.M., Alghamdi, S., Alsomali, M., Ahmed, A.O., Portelli, S., Oppong, Y., Alves, A., Bessa, T.B., Campino, S., Caws, M., Chatterjee, A., Crampin, A.C., Dheda, K., Furnham, N., Glynn, J.R., Grandjean, L.,

Minh Ha, D., Hasan, R., Hasan, Z., Hibberd, M.L., Joloba, M., Jones-López, E.C., Matsumoto, T., Miranda, A., Moore, D.J., Mocillo, N., Panaiotov, S., Parkhill, J., Penha, C., Perdigão, J., Portugal, I., Rchiad, Z., Robledo, J., Sheen, P., Shesha, N.T., Sirgel, F.A., Sola, C., Oliveira Sousa, E., Streicher, E.M., Helden, P. Van, Viveiros, M., Warren, R.M., McNerney, R., Pain, A., Clark, T.G. 2018. Genome-wide analysis of multi- and extensively drug-resistant *Mycobacterium tuberculosis*. *Nat Genet 40*, 307-316.

Doi, Y., Wachino, J. ichi, Arakawa, Y. 2016. Aminoglycoside resistance: The emergence of acquired 16S ribosomal RNA methyltransferases. *Infect Dis Clin North Am 30*, 523-537.

Ellington, M.J., Ekelund, O., Aarestrup, F.M., Canton, R., Doumith, M., Giske, C., Grundman, H., Hasman, H., Holden, M.T.G., Hopkins, K.L., Iredell, J., Kahlmeter, G., Köser, C.U., MacGowan, A., Mevius, D., Mulvey, M., Naas, T., Peto, T., Rolain, J.M., Samuelsen, Woodford, N. 2017. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin. *Microbiol Infect 23*, 2-22.

García-Galán, A., Nouvel, L.X., Baranowski, E., Gómez-Martín, Á., Sánchez, A., Citti, C., de la Fe, C. 2020. *Mycoplasma bovis* in Spanish cattle herds: Two groups of multiresistant isolates predominate, with one remaining susceptible to fluoroquinolones. *Pathogens 9*, 545.

Gautier-Bouchardon, A. V. 2018. Antimicrobial Resistance in *Mycoplasma* spp. *Microbiol Spectr 6*.

Gautier-Bouchardon, A. V., Ferré, S., Le Grand, D., Paoli, A., Gay, E., Poumarat, F. 2014. Overall decrease in the susceptibility of *Mycoplasma bovis* to antimicrobials over the past 30 years in France. PLoS One.

Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G., 2013. QUAST: Quality assessment tool for genome assemblies. *Bioinformatics 9*, e87672.

Hata, E., Harada, T., Itoh, M. 2019. Relationship between antimicrobial susceptibility and multilocus sequence type of *Mycoplasma bovis* isolates and development of a method for rapid detection of point mutations involved in decreased susceptibility to macrolides, lincosamides, tetracyclines, and spectinomycin. *Appl Environ Microbiol 85*, e00575-19.

Jaillard, M., Lima, L., Tournoud, M., Mahé, P., van Belkum, A., Lacroix, V., Jacob, L. 2018. A fast and agnostic method for bacterial genome-wide association studies: Bridging the gap between k-mers and genetic events. *PLoS Genet 14*, e1007758.

Katoh, K., Standley, D.M. 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol Biol Evol 30*, 772-780.

Khalil, D., Becker, C.A.M., Tardy, F. 2017. Monitoring the decrease in susceptibility to ribosomal RNAs targeting antimicrobials and its molecular basis in clinical *Mycoplasma bovis* isolates over time. *Microb Drug Resist 23*, 799-811.

Khalil, D., Becker, C.A.M., Tardy, F. 2015. Alterations in the quinolone resistance-determining regions and fluoroquinolone resistance in clinical isolates and laboratory-derived mutants of *Mycoplasma bovis*: Not all genotypes may be equal. *Appl Environ Microbiol 82*, 1060–1068.

Kinnear, A., McAllister, T.A., Zaheer, R., Waldner, M., Ruzzini, A.C., Andrés-Lasheras, S., Parker, S., Hill, J.E., Jelinski, M.D. 2020. Investigation of macrolide resistance genotypes in *Mycoplasma bovis* isolates from canadian feedlot cattle. *Pathogens 9*, 622.

Klein, U., de Jong, A., Youala, M., El Garch, F., Stevenin, C., Moyaert, H., Rose, M., Catania, S., Gyuranecz, M., Pridmore, A., Ayling, R.D. 2019. New antimicrobial susceptibility data from monitoring of *Mycoplasma bovis* isolated in Europe. *Vet Microbiol 238*, 108432.

Kong, L.C., Gao, D., Jia, B.Y., Wang, Z., Gao, Y.H., Pei, Z.H., Liu, S.M., Xin, J.Q., Ma, H.X. 2016. Antimicrobial susceptibility and molecular characterization of macrolide resistance of *Mycoplasma bovis* isolates from multiple provinces in China. *J Vet Med Sci 78,* 293-296.

Koren, S., Walenz, B., Berlin, K., Miller, J., Bergman, N., Phillippy, A. 2016. Canu: scalable and accurate long-read assembly via adaptive k -mer weighting and repeat separation. *Genome Res 27*, 722-736.

Ledger, L., Eidt, J., Cai, H.Y. 2020. Identification of antimicrobial resistance-associated genes through whole genome sequencing of *Mycoplasma bovis* isolates with different antimicrobial resistances. *Pathogens 9*, 588.

Lerner, U., Amram, E., Ayling, R.D., Mikula, I., Gerchman, I., Harrus, S., Teff, D., Yogev, D., Lysnyansky, I. 2014. Acquired resistance to the 16-membered macrolides tylosin and tilmicosin by *Mycoplasma bovis*. *Vet Microbiol 168*, 365-371.

Letunic, I., Bork, P. 2019. Interactive Tree of Life (iTOL) v4: Recent updates and new developments. *Nucleic Acids Res 47*, 256-259.

Li, S.L., Sun, H.M., Zhu, B.L., Liu, F., Zhao, H.Q. 2017. Whole genome analysis reveals new insights into macrolide resistance in *Mycoplasma pneumoniae*. *Biomed Environ Sci 30*, 343-350.

Liu, M., Douthwaite, S. 2002. Resistance to the macrolide antibiotic tylosin is conferred by single methylations at 23S rRNA nucleotides G748 and A2058 acting in synergy. *Proc Natl Acad Sci USA 99*, 14658-14663.

Liu, Y., Xu, S., Li, M., Zhou, M., Huo, W., Gao, J., Liu, G., Kastelic, J.P., Han, B. 2020. Molecular characteristics and antibiotic susceptibility profiles of *Mycoplasma bovis* associated with mastitis on dairy farms in China. *Prev Vet Med 182*, 105106.

Lysnyansky, I., Ayling, R.D. 2016. *Mycoplasma bovis*: Mechanisms of resistance and trends in antimicrobial susceptibility. *Front Microbiol 27*, 595.

Lysnyansky, I., Gerchman, I., Mikula, I., Gobbo, F., Catania, S., Levisohn, S. 2013. Molecular characterization of acquired enrofloxacin resistance in *Mycoplasma synoviae* field isolates. *Antimicrob Agents Chemother 57*, 3072-3077.

Lysnyansky, I., Mikula, I., Gerchman, I., Levisohn, S. 2009. Rapid detection of a point mutation in the parC gene associated with decreased susceptibility to fluoroquinolones in *Mycoplasma bovis*. *Antimicrob Agents Chemother 53*, 4911-4914.

Maunsell, F.P., Donovan, G.A. 2009. *Mycoplasma bovis* infections in young calves. *Vet Clin North*

Am Food Anim Pract 25, 139-177.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle. *J Vet Intern Med 25*, 772-738.

Nessar, R., Reyrat, J.M., Murray, A., Gicquel, B., 2011. Genetic analysis of new 16s rRNA mutations conferring aminoglycoside resistance in *Mycobacterium abscessus*. *J Antimicrob Chemother 66*, 1719-1724.

Vereecke, N, Bokma, J., Haesebrouck, F., Nauwynck, H., Boyen, F., Pardon, B., Theuns, S. 2020. High quality genome assemblies of *Mycoplasma bovis* using a taxon-specific Bonito basecaller for MinION and Flongle long-read nanopore sequencing. *BMC Bioinformatics 21*, 517.

Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House, J.K. 2018. A review of mycoplasma diagnostics in cattle. *J Vet Intern Med 32*, 1241-1252.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W. 2015. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res 25*, 1043-1055.

Pereyre, S., Goret, J., Bébéar, C. 2016. *Mycoplasma pneumoniae*: Current knowledge on macrolide resistance and treatment. *Front Microbiol 7*, 974.

Pereyre, S., Guyot, C., Renaudin, H., Charron, A., Bébéar, C., Bébéar, C.M. 2004. In vitro selection and characterization of resistance to macrolides and related antibiotics in *Mycoplasma pneumoniae*. *Antimicrob Agents Chemother 48*, 460-465.

Prats-van der Ham, M., Tatay-Dualde, J., Gómez-Martín, Á., Corrales, J.C., Contreras, A., Sánchez, A., de la Fe, C. 2018. 23S rRNA and L22 ribosomal protein are involved in the acquisition of macrolide and lincosamide resistance in *Mycoplasma capricolum subsp. capricolum*. *Vet Microbiol 216*, 207-211.

Raherison, S., Gonzalez, P., Renaudin, H., Charron, A., Bébéar, C., Bébéar, C.M. 2002. Evidence of active efflux in resistance to ciprofloxacin and to ethidium bromide by *Mycoplasma hominis*. *Antimicrob Agents Chemother 46*, 672-679.

Razin, S., Yogev, D., Naot, Y. 1998. Molecular biology and pathogenicity of Mycoplasmas. *Microbiol. Mol. Biol. Rev. 62*, 1094-1156.

Sato, T., Higuchi, H., Yokota, S.I., Tamura, Y. 2017. *Mycoplasma bovis* isolates from dairy calves in Japan have less susceptibility than a reference strain to all approved macrolides associated with a point mutation (G748A) combined with multiple species-specific nucleotide alterations in 23S rRNA. *Microbiol Immunol 61*, 215-224.

Sato, T., Okubo, T., Usui, M., Higuchi, H., Tamura, Y. 2013. Amino acid substitutions in gyrA and parC are associated with fluoroquinolone resistance in *Mycoplasma bovis* isolates from Japanese dairy calves. *J Vet Med Sci 75,* 1063-1065.

Seemann, T. 2014. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics 30*, 2068-2069.

Sulyok, K.M., Bekő, K., Kreizinger, Z., Wehmann, E., Jerzsele, Á., Rónai, Z., Turcsányi, I., Makrai,

L., Szeredi, L., Jánosi, S., Nagy, S.Á., Gyuranecz, M. 2018. Development of molecular methods for the rapid detection of antibiotic susceptibility of *Mycoplasma bovis*. *Vet Microbiol 213*, 47-57.

Sulyok, K.M., Kreizinger, Z., Wehmann, E., Lysnyansky, I., Bányai, K., Marton, S., Jerzsele, A., Rónai, Z., Turcsányi, I., Makrai, L., Jánosi, S., Nagy, S.Á., Gyuranecz, M. 2017. Mutations associated with decreased susceptibility to seven antimicrobial families in field and laboratory-derived *Mycoplasma bovis* strains. *Antimicrob Agents Chemother 61*, e01983-16.

Tatay-Dualde, J., Prats-van der Ham, M., de la Fe, C., Paterna, A., Sánchez, A., Corrales, J.C., Contreras, A., Gómez-Martín, Á. 2017. Mutations in the quinolone resistance determining region conferring resistance to fluoroquinolones in *Mycoplasma agalactiae*. *Vet Microbiol 207,* 63-68.

World Health Organization (WHO), 2017. WHO guidelines on use of medically important antimicrobials in food-producing animals. https://www.who.int/foodsafety/publications/cia_guidelines/en/

Wood, D.E., Lu, J., Langmead, B. 2019. Improved metagenomic analysis with Kraken 2. *Genome Biol 20*, 257.

World Organization for Animal Health (OIE). 2019. OIE List of antimicrobial agents of veterinary importance. https://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_OIE_List_antimicrobials_July2019.pdf (last accessed 30 March, 2021).

CHAPTER 6

GENERAL DISCUSSION

Worldwide, in all cattle production systems, *Mycoplasma bovis* remains a huge cause of high antimicrobial use, hampering animal welfare and economic losses. To combat this highly infectious pathogen, identification, antimicrobial susceptibility testing (AST), and knowledge about its epidemiology are of great importance. In this thesis, new MALDI-TOF MS and whole genome sequencing protocols for the identification of *M. bovis* from BALf and additional AST were developed and validated. These methods and the information that came to light while using these methods can help in better understanding and controlling *M. bovis* infections.

In this general discussion, first the improvements made on *M. bovis* diagnostics with the present available techniques and their (dis)advantages will be outlined. This overview will be followed by recommendations on the diagnostic approach in (1) outbreak management of *M. bovis*, (2) purchase policies, and (3) *M. bovis* herd status. These recommendations can help veterinarians in their decision making, and other authorities in implementing (inter)national surveillance and monitoring systems for *M. bovis*.

6.1 INNOVATIONS IN *MYCOPLASMA BOVIS* DIAGNOSTICS

For a long time, the three commercially available methods to diagnose a present or recent *M. bovis* infection were culture, PCR, and antibody ELISA (Parker et al., 2018). These methods all have their potential, but also several drawbacks, like higher costs, low diagnostic accuracy, interpretative issues and a long sample-to-result turnaround time, as was extensively described in the general introduction. Although unfounded, it is mostly assumed that PCR is the best method for *M. bovis* identification, reaching almost 100% sensitivity and 100% specificity. In contrast, the selective-indicative agar, which is widely used in Belgium for the identification of *M. bovis*, is frequently criticized by international experts, because no studies showed its diagnostic potential. In this thesis, qPCR appeared less perfect as previously assumed, and the selective-indicative agar, although having a moderate sensitivity (70.5%), even showed a greater specificity (93.9%) than the triplex qPCR (88.9%). An advantage of PCR over the selective-indicative agar is that multiplex PCR can also include identification of other pathogens (*Mycoplasma* species, bacteria, viruses), and turnaround time is faster. In Fig. 1, the currently available methods for the identification of *M. bovis* from BALf are shown, including the two new techniques developed in this thesis. MALDI-TOF MS can be applied in three different ways. When the sample is cultured on conventional agar for *Mycoplasma* species (5-10 days), subsequent identification with the ‘direct transfer method’ is sometimes

possible when enough *M. bovis* material can be gathered (Pereyre et al., 2013) . However, it should be kept in mind, that sometimes false positive results (*Mycoplasma alkalescens* and *Mycoplasma arginini*) can be obtained due to interference of medium-related (*e.g.* horse serum, colistin) peaks (Chapter 3.1). When the 'direct transfer method' fails, probably due to insufficient bacterial mass, an *M. bovis* colony from the agar plate can be added to a specific liquid culture (Pereyre et al., 2013; Randall et al., 2015). After incubation of 24-120 hours in liquid culture with pyruvate, a protein extraction and MALDI-TOF MS analysis results in reliable identification of *M. bovis* (Chapter 3.2). However, these methods still require a culture step on agar taking 5-10 days before result. Therefore the method 'rapid identification of *M. bovis* with MALDI-TOF MS' (RIMM), using an enrichment procedure of 2-3 days in liquid medium, is more likely to be used in routine diagnostics when rapid identification is necessary (Chapter 3.3). In contrast to the first two methods, the latter method has only been tested on BALf. However, preliminary tests show promising results in milk as well, after only minor adjustments to the RIMM protocol. It can be expected that similar protocols will probably also work for nasal swabs or samples from other body fluids. As final method, direct identification of *M. bovis* from respiratory tract samples with nanopore sequencing (ONT) was shown to be possible (Chapter 3.4). Although ONT is less sensitive and more expensive than RIMM, this method should be able to simultaneously identify all *Mycoplasma* species, bacteria and viruses. This is in contrast to qPCR, where the target for specific *Mycoplasma* species or other micro-organisms are previously defined.

To date, no routine AST is performed in Belgium, and a reference framework for resistance determination is lacking worldwide. In this thesis a new method to determine antimicrobial susceptibility of *M. bovis* for macrolides and fluoroquinolones with nanopore sequencing was developed. In addition, this method also provides the possibility of strain typing. An important remark to make is that AST and strain typing with nanopore sequencing, currently still require the purification of high *M. bovis* DNA concentrations. These can only be obtained following culture-based methods for the moment, such as the selective-indicative agar or RIMM (Fig. 1), but this can of course change in the future with increasing technological possibilities.

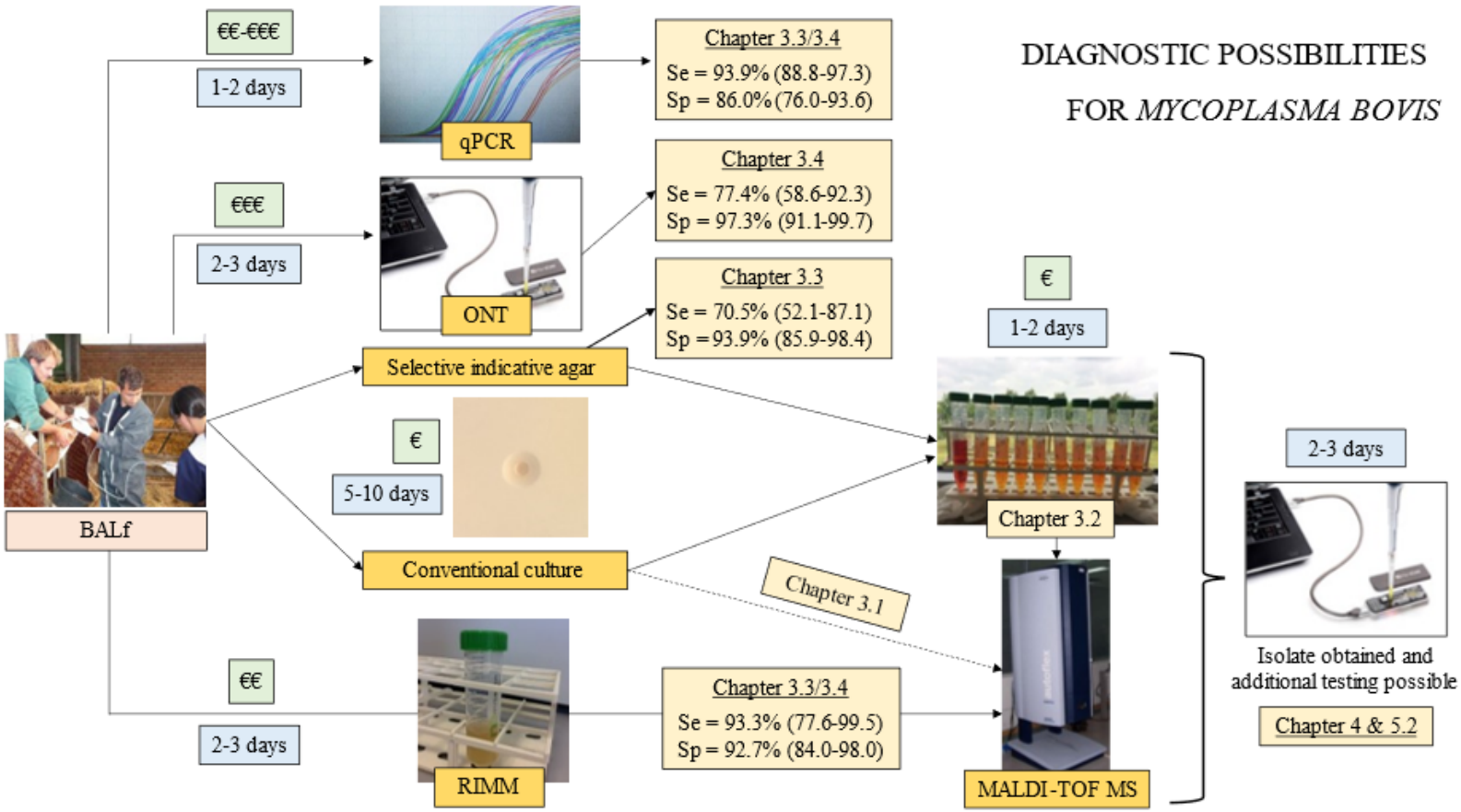


Figure 1. Currently available diagnostic methods for the identification of *M. bovis* and its cost, turnaround time and diagnostic accuracy. Cost and turnaround time are estimated, and can vary between laboratories. The dashed line represents the direct transfer method, which is not optimized (yet). ONT: nanopore sequencing, RIMM: rapid identification of *Mycoplasma bovis* with MALDI-TOF MS.

Therefore, to rapidly identify *M. bovis* we recommend the RIMM method as the most cost-effective identification method (2-3 days). Afterwards, AST (for macrolides and fluoroquinolones) and strain typing can be done with nanopore sequencing (2 days). In the next chapters, more specific recommendations and clarification will be given for specific events, such as outbreak management, purchase, and herd status determination.

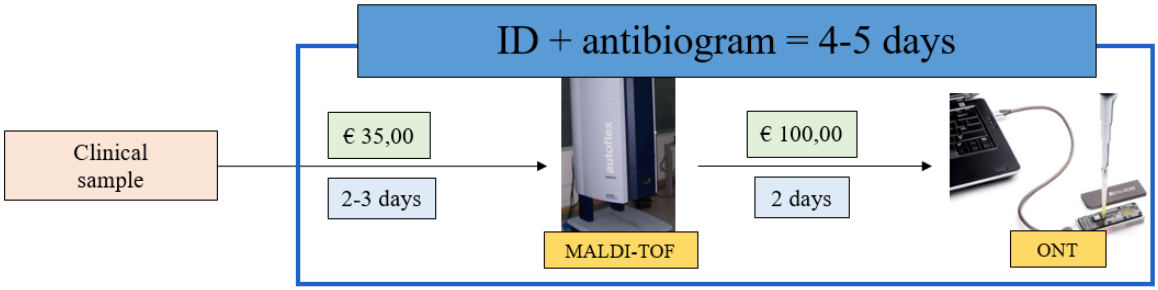


Figure 2. Recommended cost-efficient workflow for *M. bovis* identification and antimicrobial susceptibility testing. Cost and turnaround time are estimated, and can vary between laboratories

6.2 *MYCOPLASMA BOVIS* OUTBREAK MANAGEMENT

While facing a disease outbreak, both a diagnostic decision and an antimicrobial treatment decision need to be made. Preferably, before the antimicrobial treatment decision is made in a BRD or mastitis outbreak, one should diagnose whether *M. bovis* is involved or not because this has major implications for treatment and general control measures. Different diagnostic approaches are possible, and among other factors the urgency of the situation and financial limitations of the farm will drive the veterinarian to a tailored approach for the particular farm. The information from the laboratory results can aid to make a better informed choice of an antimicrobial for first intention treatment. This is especially important when considering metaphylactic treatment, as large amounts of antimicrobials will be used. Both diagnostic decision making and antimicrobial treatment decision making will be outlined beneath. In addition, the use of strain typing in outbreak management will be discussed on the basis of an example.

Diagnostic decision making

Many factors influence the veterinarian in his/her decision to sample a particular outbreak, including the management and history of the farm, the farmers expectations and financial restrictions and the suspected involved pathogen(s). A whole range of diagnostic tests is available, with advantages and disadvantages, varying accuracy and associated cost, resulting in the fact that there is no one size fits all diagnostic test. Hence, several questions arise in this diagnostic decision making helping the veterinarian to decide which test(s) to request. These questions are: (1) Which pathogens should be looked for in the laboratory for this particular outbreak? (2) Is it necessary to obtain an isolate for additional testing (*e.g.* AST, strain typing)? (3) Which samples can be obtained and are necessary for the desired analysis? (4) Is it possible to pool the samples to reduce analytic costs without losing accuracy? For each of these questions the possibilities and diagnostic methods are shown in Table 1.

As mentioned before, some qPCR and NGS methods can offer, in function of need and sometimes at higher cost, simultaneous identification of other *Mycoplasma* species, bacteria or viruses. The sample choice depends mainly on the disease form present (respiratory, mastitis, arthritis). Also, even though a good agreement between deep nasopharyngeal swabs, BAL, and TTA for *M. bovis* has been observed (Doyle et al., 2017), it should be considered that for example deep nasopharyngeal swabs may be easy to obtain, but compared to BALf/TTA/TTW have an increased risk of overgrowth of other pathogens and/or contamination (Van Driessche et al., 2017). In addition, not all methods are already optimized for all possible samples (Table 1). Pooling of samples can seriously reduce cost, especially for costly methods such as qPCR or nanopore sequencing. Taking five samples for pooling appeared the most cost-effective number of analyzing samples with a diagnostic test (sensitivity: 70%; specificity: 100%) (Pardon and Buczinski, 2020). In this case the risk of not finding the pathogen causing the outbreak is almost zero, if the test would be 100% specific (Pardon and Buczinski, 2020). We showed that pooling five BALf samples is indeed a reliable *M. bovis* identification method when applied with the RIMM and nanopore sequencing (Chapter 3.4). The latter also showed good concordance for viruses (unpublished results).

Table 1. Questions to be asked in the diagnostic decision process during a potential *M. bovis* outbreak.

	Culture	*RIMM*	*qPCR*	*Nanopore sequencing*
1. Which pathogens do I want to detect?				
M. bovis	✓	✓	✓	✓
M. bovis + bacteria	✓[a]	-	✓	±
M. bovis + viruses	-	-	✓	✓
2. Do I want to perform additional antimicrobial susceptibility testing or strain typing?				
Isolate obtained	✓	✓	-	-
3. What sample do I want to analyze to detect *M. bovis*?				
BALf/TTA	✓	✓	✓	✓
Deep nasal swab	✓	ND	✓	✓
Milk	✓	±	✓	ND
Body fluids*	✓	ND	✓	✓
4. Do I want to pool five samples for *M. bovis* identification?				
BALf/TTA	ND	✓	✓	✓
Deep nasal swab	ND	ND	✓	✓
Milk	ND	ND	✓	ND
Body fluids*	ND	ND	ND	ND

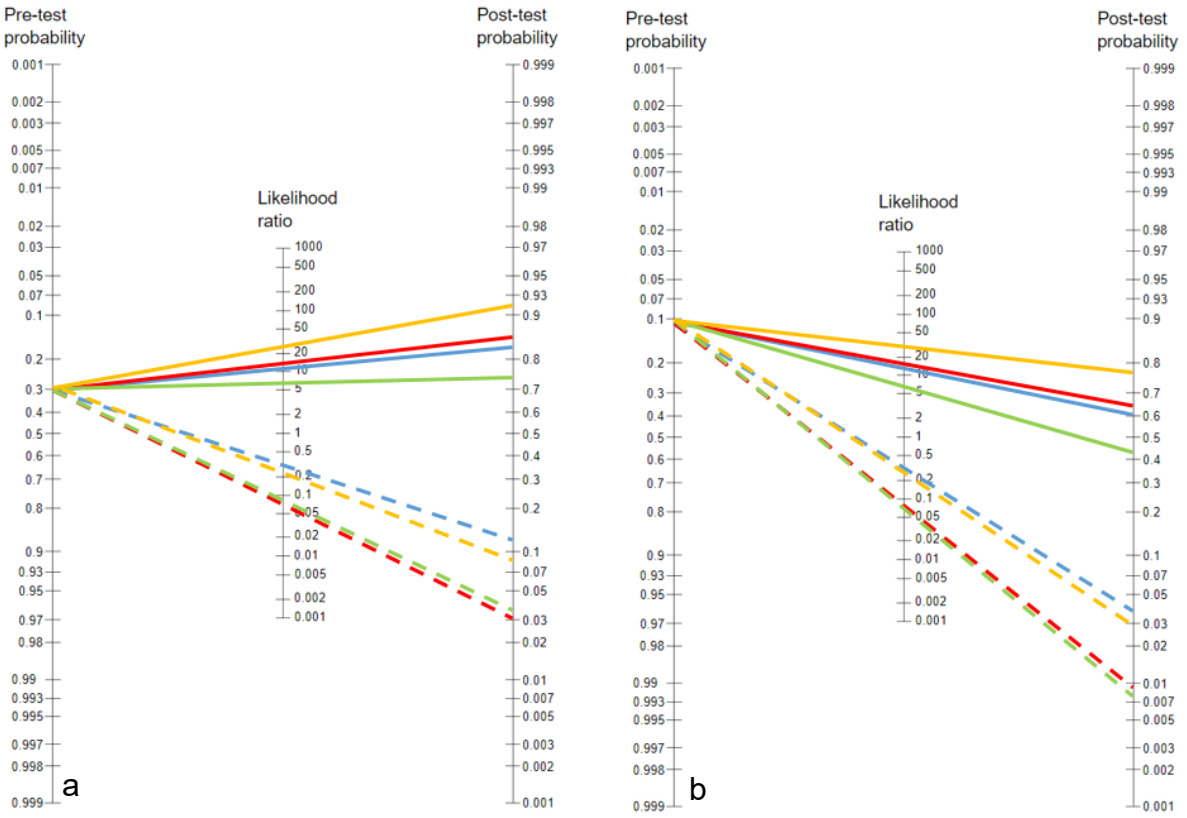
[a] additional cost for bacteria * *e.g.* joint, pleural, abdominal or cerebrospinal fluid; ± probably after small adjustments to the protocol; ND: not determined

Next to the above mentioned considerations, turnaround time and cost-effectiveness of the different tests are very important in the selection of a diagnostic method (Fig 1.). However, interpretation of 'accuracy' of a test is sometimes a difficult concept to comprehend. Therefore, Fagan nomograms can help to visualize what the sensitivity and specificity of a test actually mean in the field and how they contribute to the relevance of the test outcome (Caraguel and Vanderstichel, 2013). First sensitivity and specificity are translated to positive and negative likelihood ratios (LR) per diagnostic test (LR+ = Se/(1-Sp); LR- = (1-Se)/Sp) (Caraguel and Vanderstichel, 2013). These values support and give direction to the translation from pre-test probability (possibility that an animal is infected, mostly based on prevalence) to the post-test probability (possibility that indeed the animal is infected when the test is either positive or negative). When LR is smaller than 1, the test result supports the absence of the condition. When the LR is 1, the test has no diagnostic value, and when the LR is higher than 1, the presence of the condition is supported by the test. This may sound too complicated to bring into practice, but available online calculators (http://araw.mede.uic.edu/cgi-bin/testcalc.pl) or the two-step Fagan's nomogram can be used on paper without additional calculations (Caraguel and Vanderstichel, 2013) for the visualization of these outcomes.

For example, in an acute outbreak of BRD on a beef farm in Belgium, the prevalence of *M. bovis* is approximately 30% (Pardon et al., 2020). Therefore, a reasonable estimate of our pre-

test probability, the possibility that an animal is infected with *M. bovis*, would be 0.3. Using different diagnostic methods, with different sensitivity and specificity for *M. bovis* identification, will eventually lead to different post-test probabilities, as shown in Fig. 3a. When the test result in the lab is positive, the chance an animal is truly infected with *M. bovis,* depending on the used method, is 75-92% (post-test probability/positive predictive value) when a prevalence of 30% is expected. A negative lab test means that the chance the animal is infected with *M. bovis* is still 3-12% (post-test probability; 1-negative predictive value), depending on the used test. Probably, while facing an acute BRD outbreak, this is an acceptable risk, especially when as recommended at least five animals are tested to obtain a farm-level result (Pardon and Buczinski, 2020). Both false positive and false negative results have their consequences. On the one hand, animal welfare will be hampered when an animal is not properly treated although enduring a bacterial infection (false negative). On the other hand, the risk of antimicrobial resistance will increase when animals not infected are treated with antimicrobials (false positive). Figure 2a shows that when comparing these tests, the lowest probability for a false positive test is the use of nanopore sequencing (orange), while the lowest probability of false negatives can be obtained by RIMM (red) or qPCR (green). When prevalence of *M. bovis* is believed to be lower in another country or when eradication programs are implemented (a lower prevalence would be expected then, *e.g.* 10%), this can influence the post-test probability as shown in Fig 3b.

Nevertheless, for a reliable analysis and interpretation of the test results in a disease outbreak, several basic rules in sample-taking should be considered. First, the sampled animal should: (1) reflect the (herd) problem, (2) be in the acute phase of the disease, and (3) not previously be treated with antimicrobials. Next, the sample should be taken as clean as possible (especially for culture-based methods) from the site of interest, and finally the samples should be stored and transported at low temperatures. For *M. bovis* and *Pasteurellaceae* from BALf, the recommended temperature is 0-8ºC (Boonyayatra et al., 2010; Parker et al., 2016; Van Driessche et al., 2020). Freezing of samples is possible and recommended for culture when arrival in the laboratory takes more than 24 hours after sampling, however a decrease in sensitivity can be expected (Biddle et al., 2004; Boonyayatra et al., 2010; Vyletělová, 2010; Gille et al., 2018). Freezing is discouraged for identification of *M. bovis* from BALf with nanopore sequencing, as non-viable *M. bovis* are not detected, due to the nuclease step in the protocol, resulting in a lower sensitivity.



Diagnostic method	Sensitivity	Specificity	LR+ (full line)	LR- (dashed line)
Culture (blue)	0.705	0.939	12	0.31
RIMM (red)	0.933	0.927	13	0.07
qPCR (green)	0.939	0.860	6.71	0.07
Nanopore (orange)	0.774	0.973	29	0.23

Figure 3. Example of Fagan nomograms showing the pre- and post-test probability of culture (blue), RIMM (red), qPCR (green), and nanopore sequencing (orange) on BALf, facing an acute outbreak of bovine respiratory disease while prevalence is expected to be 30% (a) or 10% (b). Sensitivity, specificity, and corresponding positive and negative likelihood ratios (LR) per diagnostic test are shown in the table.

Antimicrobial treatment decision making

Once diagnostic samples are taken, the decision to either start or wait with antimicrobial therapy before the results of the diagnostic tests get back, needs to be made. Rapid identification of *M. bovis*, can already direct towards a more appropriate first choice antimicrobial use, as its inherent resistance can be circumvented. A combination of experience on farm therapy success and local antimicrobial susceptibility data, can then help in the antimicrobial choice. For example, in Belgium we did not observe acquired *in vitro* antimicrobial resistance against tetracyclines in *M. bovis*, and only very limited acquired resistance against florfenicol (Chapter 5.1). In addition, it was also shown in an *M. bovis* field

trial that parenteral use with these antimicrobials resulted in a rapid cure of almost all clinically affected animals (De Cremer, 2019). Nevertheless, it is still possible that resistance against these antimicrobials in *M. bovis* occurs and may result in therapy failure. In this case, a second antimicrobial should be administered. In the authors opinion, it is important to already have an antibiogram before initiation of a second therapy, because in Belgium, a fair level of acquired resistance against macrolides and fluoroquinolones has been observed (Chapter 5.1). Therefore, blind usage of gamithromycin, for which 50% of the Belgian *M. bovis* isolates demonstrated acquired resistance, and with even greater odds of acquired resistance in beef cattle, would signify an unnecessary delay in treatment. The longer the period before appropriate treatment is implemented for *M. bovis*, the higher the risk that an animal becomes chronically diseased as a result of immune evasion, virulence factors or biofilm formation of *M. bovis* (Buchenau et al. 2010; Razin and Hayflick, 2010; Perez-Casal, 2020). Hence, determining antimicrobial susceptibility of *M. bovis* for macrolides and fluoroquinolones with nanopore sequencing can be of great help (Chapter 5.2). The identification of specific mutations associated with the step-wise fluoroquinolone resistance is of great importance. Using fluoroquinolones on a farm with animals harbouring *M. bovis* strains with only a slight increase in fluoroquinolone MIC value, increases the risk of an additional mutation, resulting in clinically relevant resistance of *M. bovis*. Logically, when these specific step-wise mutations are observed, the use of fluoroquinolones should definitely be discouraged.

Therefore, the authors recommend in BRD outbreaks with suspected *M. bovis* involvement, to obtain BALf, process with RIMM (2-3 days), and when positive for *M. bovis*, perform additional AST with nanopore sequencing (2 days) (Fig 2). Also, we would recommend systematic sampling and laboratory testing in every situation that may need a group antimicrobial therapy.

For mastitis cases, antimicrobial therapy is not recommended, and therefore the above suggested workflow may seem less attractive (Jasper, 1981; Bushnell, 1984). However, it is really important to remember that in mastitis outbreaks where *M. bovis* is involved, calves and young stock are frequently affected as well, and may need antimicrobial treatment. For example, in 15 of 17 Finnish outbreaks starting with a mastitis index case, *M. bovis* could be isolated from nasal swabs in the calves (Vähänikkilä et al., 2019). In addition, a high prevalence of *M. bovis* was seen in calves in herds with previous *M. bovis* outbreaks (Maunsell and Donovan, 2009). But also when no clinical mastitis is detected in the last few

months, isolation of *M. bovis* in calves is possible, as half a year later in 10 out of these 17 previous herds, calves were still positive. One and 1.5 year after the index case, *M. bovis* was still present in the calves on 3 of these herds (Vähänikkilä et al., 2019).

MIC determination can also be applied after an isolate is obtained, as commercially offered in the Netherlands. Alternatively, qPCR based methods (Melt-MAMA, HRM) are offered to motivated Hungarian cattle-breeders in a research setting. These qPCR methods can also be applied directly to clinical samples for most antimicrobials. However, resistance genes can easily be overlooked, as only most common (in a certain region) SNPs are targeted. When communicating AST results to veterinarians and farmers, it is of huge importance to warn them about the limitations of the used tests. Especially, the lack of specific clinical breakpoints for *M. bovis* can result in unintentional, but inappropriate antimicrobial use.

Use of strain typing in M. bovis *outbreak management*

In 2020, our veterinary teaching hospital was confronted with an acute outbreak of respiratory disease and otitis in a large beef herd (~100 calves). *M. bovis* was isolated from BALf, nasal swabs, and the middle ear. The same herd also faced respiratory disease in 2017 caused by *M. bovis* as well. Following AST protocols and ECOFFs as determined in Chapter 5.1, four isolates (2 from 2017; 2 from 2020) were wild type for florfenicol, tetracyclines, gamithromycin and enrofloxacin. Indeed, in the first outbreak, immediate treatment with either florfenicol or oxytetracycline resulted in cure of almost all animals after 8 and 14 days as evidenced by thoracic ultrasonography, respectively (De Cremer, 2019). Animals that did not cure (7/161), were isolated from the herd and eventually culled. However, in the second outbreak (2020), the cure rate to antimicrobial treatment (florfenicol, oxytetracycline, gamithromycin, enrofloxacin) was much lower, and after 21 days of treatment, still 30% (28/94) of the animals showed lung lesions on thoracic ultrasonography. The question was raised whether this was a relapse of a persisting strain or a new outbreak resulting from a breach in external biosecurity of this farm. To answer this question, the two isolates obtained in 2017 and 2020 were cultured, sequenced, and strain typed as described in Chapter 4.

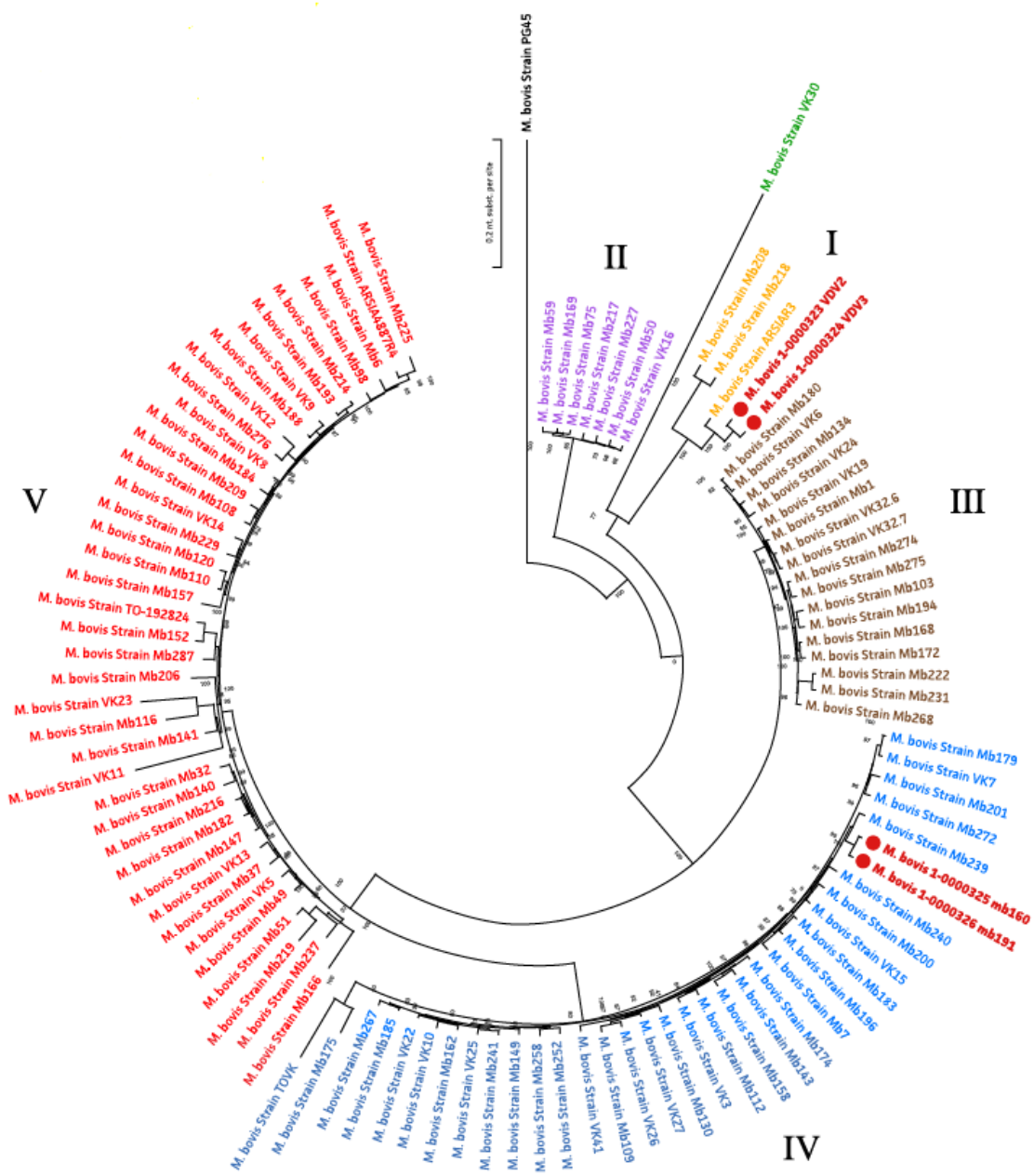


Figure 4. Phylogenomic analysis of 4 *M. bovis* strains (bold, red) obtained from two *M. bovis* outbreaks on a Belgian beef farm. The first outbreak (2017) was represented by a *M. bovis* strain from Belgian genomic cluster IV, while those from the second outbreak (2020) were closely related to strains from the genomic cluster I (Chapter 4).

The phylogenomic analysis showed that both acute outbreaks were caused by *M. bovis* strains belonging to different Belgian genomic clusters (Fig 4; bold red). Therefore, it seems very likely that a new *M. bovis* strain was introduced in the herd, caused by a security breach. The breach in biosecurity was not immediately detected, however when in the future more *M.*

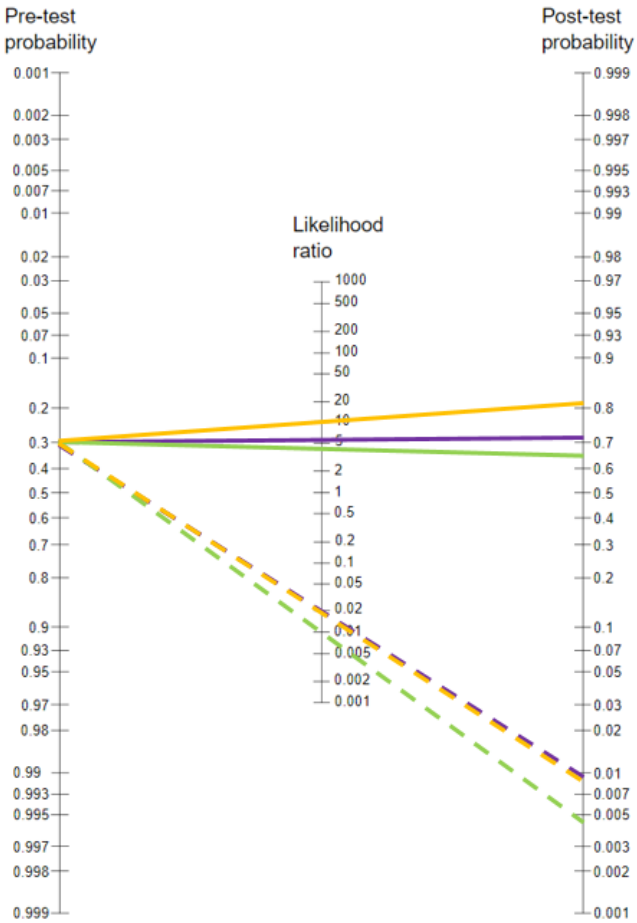
bovis strains are systematically collected and sequenced, tracing back the source might be more feasible. The poor therapy response in 2020 of the *M. bovis* strain belonging to the first Belgian genomic cluster, may be due to a late detection of the involvement of *M. bovis* in the BRD outbreak, which emphasizes the importance of rapid diagnosis. In addition, poor treatment success could also have been due to a more virulent *M. bovis* strain or undetected other pathogens. Potentially, this strain improved its characteristics to evade antimicrobials or produced biofilms better than others. More research is necessary to identify associations between strain type and virulence.

In conclusion, the diagnostic decision of using culture-based methods followed by nanopore sequencing in *M. bovis* outbreaks, can help at different moments in the antimicrobial treatment decision making process (first intention treatment, and when therapy failure occurs). In addition, the confirmation that a new strain has been introduced into the herd by strain typing, can help the veterinarian and farmer to discover possible biosecurity breaches, such as purchase, artificial insemination of infected semen, or transmission by fomites (Gonzalez et al., 1992; Haapala et al., 2018; Pardon et al., 2020). In the future, strain typing can also help to determine whether a certain strain is originally from a specific herd. An outbreak after purchase, which can be tracked back to one farm, can result in targeted protocols, tracing animals at risk and test these animals for potential follow up measurements.

6.3 USE OF *MYCOPLASMA BOVIS* DIAGNOSTICS IN PURCHASE POLICY

With purchase being the most frequently observed risk factor for the introduction of *M. bovis* into the herd, the identification of *M. bovis* carrier animals at purchase is very important. As shown with the Fagan's monogram (Figure 3), the risk that an animal is infected with *M. bovis* during an outbreak with current diagnostic methods would still be ~10% when negative test results are obtained. This may be acceptable for *M. bovis* outbreak management, as more than one animal will be tested and the pathogen is already circulating in the herd. However, to prevent *M. bovis* from entering a negative herd, a risk of 10% with every purchase the farmer makes is obviously too high. To reduce false negative outcomes, the sensitivity of a test is very important. However, when one test cannot reach the desired sensitivity (as shown above), parallel testing can help to increase sensitivity. Therefore, parallel testing may be a solution in purchase protocols, while keeping the animal in quarantine awaiting the results. Examples of different parallel testing options for the identification of *M. bovis* on BALf are

given in Fig 5. In general, when testing two methods in parallel, the Fagan nomogram shows that the chance for a false negative result is now smaller than 1%. Parallel use of RIMM and qPCR even results in a risk of 0.5%. This risk would be more acceptable in purchase protocols.



Diagnostic method	Sensitivity	Specificity	LR+ (full line)	LR- (dashed line)
Culture + qPCR (purple)	0.982	0.808	5.11	0.02
RIMM + qPCR (green)	0.996	0.797	4.91	0.01
RIMM + nanopore (orange)	0.985	0.902	10	0.02

Figure 5. Fagan nomogram showing the pre- and post-test probability of parallel testing of culture, RIMM, qPCR, and nanopore sequencing on BALf, when prevalence of *M. bovis* is 30%. Sensitivity (Se), specificity (Sp), and corresponding positive and negative likelihood ratios (LR) per diagnostic test are shown in the table. Se and Sp determined by Winepsicope (multiple diagnostic tests: http://www.winepi.net/uk/index.htm). Population size was set at 100.

One of the downfalls at the moment is that the diagnostic accuracy of most of these methods, is only validated on BALf so far. In the context of purchase, testing on deep nasal swabs and/or milk samples may be more appropriate. This is an easier way to test animals, and it

was also shown by Hazelton et al. (2020) that colonization of the nose is most prevalent. Another sample site that can be considered, although tougher to swab, are the pharyngeal and palatine tonsils (Maunsell et al., 20212). Buckle et al. (2020) showed that sensitivity of mucosal swabs from the palatine tonsillar crypt was seven times higher than those from the bronchial main stem after slaughter of 51 asymptomatic calves. However, this kind of sample is less evident and more research (*e.g.* comparison between nasal and tonsil swabs) is necessary before implementation in purchase policies can be recommended. When the best sampling method is determined, also the diagnostic test accuracy for the different methods can be further explored.

A second downfall is the potential intermittent shedding of *M. bovis* (Caswell and Archambault, 2007; Byrne et al., 2005). A diagnostic test can be perfect, but if the animal is not shedding the pathogen, the test cannot detect it. Nevertheless, as it is suspected that animals usually start shedding after stress (*e.g.* calving, disease, transport, introduction in new herd), they probably will start shedding after purchase. Therefore, quarantine of purchased animals is of great importance, and multiple testing seems appropriate.

To counteract the absence of shedding, antibody ELISA testing could be used as an additional test. Unfortunately, these tests cannot distinguish between present, active infection (acute or chronic) or cured infection and therefore cannot distinguish between carrier animals or those that cleared the infection. It does however inform on previous contact with *M. bovis* in the herd. Possibly paired sera can give more insight, as Vähänikkilä et al. (2019) observed a downward line in serum antibody concentrations in young stock where infection seemed under control and no more *M. bovis* was detected in the second year. In contrast, 60-80% of the young stock animals in herds where *M. bovis* kept circulating remained seropositive with MilA ELISA (Vähänikkilä et al., 2019). This was in contrast to BioX ELISA, which did not follow similar patterns as culture or PCR, possibly on the one hand due to lower sensitivity of BioX ELISA compared to MilA, and on the other hand due to the low prevalence of positive animals in the BioX ELISA category. The same actually applies to qPCR, as qPCR can detect non-viable *M. bovis* as well. It is not clear how long after clearance of the infection, an animal stays positive for *M. bovis* on qPCR. In a study on veal calves 49% of the calves were still positive with qPCR four weeks after treatment with mostly macrolides or tetracyclines, while only 19% of them were still positive with culture (Becker et al., 2020). Another possibility could be a lower detection limit of qPCR compared to culture (Hazelton et al., 2018) or the inhibition of growth with culture. Although nanopore sequencing has a lower sensitivity for

M. bovis identification, it can distinguish better between presence of viable and non-viable *M. bovis*. To be entirely sure, culture remains the 'gold standard' to detect viable *M. bovis*.

6.4 ERADICATION OR HERD STATUS CERTIFICATES

Although this thesis did not focus on the diagnosis of *M. bovis* on herd level and methods were only validated on the individual animal level, the herd level status of *M. bovis* is very important. In New Zealand for example, the most recent country where *M. bovis* was introduced, started an eradication program in 2018 based on positive and negative *M. bovis* herds. Complete culling of positive herds is an internationally closely followed (and criticized) part of the program.

In this situation of obliged culling, a false positive result can have disastrous consequences on economy, animal welfare, and the farmers wellbeing. Therefore, the specificity of the used diagnostic method should approach 100% to minimize the risk of false positives. Next to false positive results, a false negative result can have enormous consequences as well. When eradicating and missing a single case, this can set the whole country back to where it started. As there are no tests available approaching 100% sensitivity and specificity, serial testing is recommended to increase in the first place specificity. In theory, first the most sensitive technique should be applied as a screening test to maximize sensitivity. In New Zealand, this would be qPCR. Also, screening all animals with qPCR will be a costly event. Therefore, antibody ELISA is often used as a cheap screening method. Unfortunately, sensitivity and specificity of ELISA tests have a very broad range, depending both on the commercial or in-house kit and samples used (Nielsen et al., 2015; Wawegama et al., 2016; Andersson et al., 2019). Therefore, combination of antibody ELISA with another method for optimal monitoring and surveillance is recommended (Vähänikkila et al., 2019). In New Zealand, this approach was also first chosen for the eradication program, namely the use of antibody ELISA (first Bio-X, for a short while Biovet and eventually the IDvet kit), and subsequent PCR (Ministry of Primary Industries, 2019). At least 100 animals were tested with ELISA on blood, and additionally PCR testing was performed to confirm an infected herd. When only one animal was positive in two consecutive ELISA tests and/or PCR, the herd was culled (Jordan et al., 2021). More recently, the strategy was modified. Now also herds are culled only based on IDvet ELISA test results. The reason is the believed high sensitivity (93.5%) and specificity (98.6%) of this antibody ELISA test (Andersson et al., 2019; Ministry of Primary Industries, 2019). It seems that a herd is categorized as infected when ≥ 30% of the

animals are seropositive, and negative as <5% is positive with antibody ELISA, due to cross-reaction with other *Mycoplasma* species. At the moment (1st of April, 2021), 262 herds in New Zealand were confirmed to be infected with *M. bovis* (67 dairy, 139 beef, 56 other) (number of total herds: ~30.000; Stats, 2019), 170,486 animals were culled, 2,030,762 tests were executed, and an investment of approximately 200 million New Zealand Dollar was made so far (Ministry for Primary Industries, 2021). Although eradication of *M. bovis* is not yet accomplished, progress towards eradication has been made. Since eradication was started in 2018, the reproduction number (R value) was always beneath 1, and only few new cases were identified in 2019 (Jordan et al., 2021). Nevertheless, *M. bovis* is still present, and small breaches in the track an tracing systems, suboptimal detection systems or a mutation resulting in increased virulence, can result in new outbreaks.

Such eradication programs are probably not applicable in Belgium or even in Europe. The prevalence of *M. bovis* is already high and in many areas already endemic (Maunsell et al., 2011; Timonen et al., 2020). In addition, the control on import and export of animals is more difficult in the European mainland than on an island, like New Zealand. Investments to pay off farmers, would be incredibly high. However, what could be an opportunity for the future in endemic regions is the implementation of *M. bovis* herd status certificates. Farmers can decide for themselves whether to invest in measurements to keep or reach a negative herd status. To free a herd of *M. bovis*, frequent testing and isolating carrier animals will be key. Return on investment will depend on the previous status of the herd, but will probably show in increased marked value of the animals, reduced antimicrobial use, and less economic losses.

How to obtain a negative herd after an outbreak, is outside the scope of this thesis. Nevertheless, metaphylactic treatment after the diagnosis of *M. bovis* and subsequent isolating or culling of chronically infected animals may be recommended. Also management changes such as termination of raw milk feeding, and individual housing until 8 weeks old could be recommended in calves. When facing severe *M. bovis* mastitis, culling is an easy decision given the poor prognosis, but the decision becomes a lot harder for subclinical mastitis. How cattle eliminate *M. bovis* is unclear, but an observational study showed that culling did not result in a faster clearance of *M. bovis* from the herd (Punyapornwithaya et al., 2012). There was however no information available on clinical status of the animals or reason for culling on the farms. Some of the farmers might have culled *M. bovis* positive animals

unintentionally, because of other reasons (*e.g.* age, non-mycoplasmal diseases). On the other hand, there are studies showing continuing presence of the same *M. bovis* strain in herds for years (Spergser et al., 2013; Parker et al., 2016). Therefore, whether animals should be culled or isolated is not clear. For dairy herds, milking suspected animals last or using a separate milking machine are logical measures, but actually putting clinically affected animals immediately in complete quarantine, avoiding contact with animals with non-*M. bovis* related diseases in the hospital pen, is likely more efficient, but not always easy to implement in practice (Punyapornwithaya et al., 2011). Further research on how to identify and isolate carrier animals is necessary to obtain a negative herd status. The new methods developed in this thesis may be used as supportive tools, but testing on herd level (*e.g.* BTM in lactating cows, nasal swabs in non-lactating cattle), next to the individual level, and frequently testing compared to single testing, should be first validated. It has already been shown that PCR on BTM can be negative, while there were still individual animals positive for *M. bovis* mastitis in the herd (Vähänikkila et al., 2019). Murai et al. (2014) showed the cost-effectiveness of eleven diagnostic strategies based on qPCR and bacterial culture to identify *M. bovis* mastitis cases. The most cost-effective method was pooling of 5 samples for culture, followed by culture on individual samples. Including qPCR on 50 or 100 pooled samples as first step, was the second most cost-effective alternative compared to the reference strategy (individual culture on all animals).

Antibody ELISA can be useful for monitoring programs, but when a farm is suspected to be positive for *M. bovis* based on antibody ELISA, it is necessary to demonstrate the presence of the pathogen by culture-based methods, before culling. Strain typing could be helpful both in *M. bovis* outbreak management, as in surveillance programs. Also *M. bovis* introduction from other countries can be traced, when more whole genomes become available (Chapter 4).

6.5 FUTURE PROSPECTS

This thesis added important information on *M. bovis* diagnostics and epidemiology, but unfortunately there are still many knowledge gaps. One of the most important research question is probably still: "How do we identify carrier animals to prevent *M. bovis* from entering the herd?". To answer this question and to establish safe protocols, more research on which samples to collect and how frequently animals should be sampled to counter intermittent shedding is necessary.

To what extend the identification and AST methods developed in this thesis can be extrapolated or adjusted to other *Mycoplasma* species, such as *M. ovipneumoniae*, *M. hyopneumoniae,* and *M. pneumoniae,* should be explored in further research. On AST, consensus on standard protocols and the determination of clinical breakpoints is important. Genetic molecular methods are showing potential, but are still only based on *in vitro* results. When clinical breakpoints cannot be realized, randomized clinical trials with known *M. bovis* strains and potential resistance genes may be an option. Until then, phenotypic and genetic molecular methods should be used in parallel to avoid missing new antimicrobial resistant mechanisms. There is minimal discussion whether antimicrobial treatment is necessary when culture shows positive results for *M. bovis*. However, interpretation of borderline qPCR and deep sequencing outcomes in association with clinical status and/or recovery phase of the calf is unclear and can have serious consequences on antimicrobial treatment, purchase, and culling. When the association between quantification of diagnostic methods and clinical status of the animal is clarified, these methods may even support the evaluation of antimicrobial treatment efficacy or prognosis in acute or chronic bacterial infections in the future.

The general public is also getting more and more concerned with animal welfare. For example, many people experienced the unpleasant or sometimes painful feeling while they were sampled for COVID-19 testing, which might result in strong opinions on performing deep nasal swabs, TTA and BAL in calves. Therefore, future research may include parameters on stress and pain levels of animals while undergoing different sampling methods, showing the combination of the most animal friendly and efficient sampling method. Also, together with antimicrobial therapy, it would be helpful to determine whether NSAIDs relieve pain when facing disease or whether these substances interfere with the cure rate of cattle infected with *M. bovis*, possibly reducing animal welfare on the long term, as was shown in preliminary research (Dudek et al., 2019).

CONCLUSION

The developed MALDI-TOF MS and ONT methods for rapid identification, antimicrobial susceptibility testing and strain typing of *M. bovis* from BALf are complementary and immediately applicable in peripheral laboratories. They can contribute to more efficient treatment of acute outbreaks and plans for *M. bovis* control or eradication. The RIMM is a cost-efficient rapid method to identify *M. bovis* and simultaneously obtaining an isolate for further (ONT) testing. NGS shows great potential for a (rapid) detection of *M. bovis* mutations associated with acquired antibiotic resistance, but further development is needed for broader use, given that not all resistance phenotypes were present in our dataset. The development of standard protocols, clinical breakpoints, and the *in vivo* validation of these *in vitro* results will still be necessary. Complementing the available whole genome sequences of more *M. bovis* isolates worldwide can support this, but can also give more insights in the epidemiology of *M. bovis* and support in track and tracing of *M. bovis* introductions and outbreaks.

REFERENCES

Andersson, A.M., Aspán, A., Wisselink, H.J., Smid, B., Ridley, A., Pelkonen, S., Autio, T., Lauritsen, K.T., Kensø, J., Gaurivaud, P., Tardy, F. 2019. A European inter-laboratory trial to evaluate the performance of three serological methods for diagnosis of *Mycoplasma bovis* infection in cattle using latent class analysis. *BMC Vet Res 15*, 369.

Becker, C.A.M., Ambroset, C., Huleux, A., Vialatte, A., Colin, A., Tricot, A., Arcangioli, M.A., Tardy, F. 2020. Monitoring *Mycoplasma bovis* diversity and antimicrobial susceptibility in calf feedlots undergoing a respiratory disease outbreak. *Pathogens 9*, 593.

Biddle, M.K., Fox, L.K., Hancock, D.D., Gaskins, C.T., Evans, M.A. 2004. Effects of storage time and thawing methods on the recovery of *Mycoplasma* species in milk samples from cows with intramammary infections. *J Dairy Sci 87,* 933-936.

Boonyayatra, S., Fox, L.K., Besser, T.E., Sawant, A., Gay, J.M. 2010. Effects of storage methods on the recovery of *Mycoplasma* species from milk samples. *Vet Microbiol 144,* 210-213.

Buchenau, I., Poumarat, F., Grand, D. Le, Linkner, H., Rosengarten, R., Hewicker-Trautwein, M. 2010. Expression of *Mycoplasma bovis* variable surface membrane proteins in the respiratory tract of calves after experimental infection with a clonal variant of *Mycoplasma bovis* type strain PG45. *Res Vet Sci 89*, 223-229.

Buckle, K.N., Castillo-Alcala, F., Ha, H., Begg, D., McDonald, W., Bingham, P. 2020. First report of the use of mucosal swabs of the palatine tonsillar crypt for detection of *Mycoplasma bovis* in naturally infected calves. *NZ Vet J 68*, 309-312.

Bushnell, R.B. 1984. Mycoplasma mastitis. *Vet Clin North Am Large Anim Pract 6*, 301-312.

Byrne, W., Markey, B., McCormack, R., Egan, J., Ball, H., Sachse, K. 2005. Persistence of *Mycoplasma bovis* infection in the mammary glands of lactating cows inoculated experimentally. *Vet Rec 156*, 767-771.

Caraguel, C.G.B., Vanderstichel, R. 2013. The two-step Fagan's nomogram: Ad hoc interpretation of a diagnostic test result without calculation. *Evid Based Med 18*, 125-128.

Caswell, J.L., Archambault, M. 2007. *Mycoplasma bovis* pneumonia in cattle. *Anim Health Res Rev 8*, 161-86.

De Cremer, L., van Leenen, K., Van Driessche, L., Bokma, J., Deprez, P., Pardon, B. 2019. Lung ultrasonography to reduce antimicrobial therapy length in an outbreak of *Mycoplasma bovis*. European Bovine Congress, 11-13 September, 's-Hertogenbosch, the Netherlands.

Doyle, D., Credille, B., Lehenbauer, T.W., Berghaus, R., Aly, S.S., Champagne, J., Blanchard, P., Crossley, B., Berghaus, L., Cochran, S., Woolums, A. 2017. Agreement among 4 sampling methods to identify respiratory disease. *J Vet Intern Med 31*, 954-959.

Dudek, K., Bednarek, D., Ayling, R.D., Kycko, A., Reichert, M. 2019. Preliminary study on the effects of enrofloxacin, flunixin meglumine and pegbovigrastim on *Mycoplasma bovis*

pneumonia. *BMC Vet Res 15*, 371.

Gille, L., Boyen, F., Van Driessche, L., Valgaeren, B., Haesebrouck, F., Deprez, P., Pardon, B. 2018. Short communication: Effect of freezer storage time and thawing method on the recovery of *Mycoplasma bovis* from bovine colostrum. *J Dairy Sci 101,* 609-613.

Gonzalez, R.N., Sears, P.M., Merrill, R.A., Hayes, G.L. 1992. Mastitis due to Mycoplasma in the state of New York during the period 1972-1990. *Cornell Vet 82*, 29-40.

Haapala, V., Pohjanvirta, T., Vähänikkilä, N., Halkilahti, J., Simonen, H., Pelkonen, S., Soveri, T., Simojoki, H., Autio, T. 2018. Semen as a source of *Mycoplasma bovis* mastitis in dairy herds. *Vet Microbiol 216*, 60-66.

Hazelton, M.S., Morton, J.M., Parker, A.M., Bosward, K.L., Sheehy, P.A., Dwyer, C.J., Niven, P.G., House, J.K. 2020. *Mycoplasma bovis* and other Mollicutes in replacement dairy heifers from *Mycoplasma bovis*-infected and uninfected herds: A 2-year longitudinal study. *J Dairy Sci 103*, 11844-11865.

Hazelton, M.S., Sheehy, P.A., Bosward, K.L., Parker, A.M., Morton, J.M., Dwyer, C.J., Niven, P.G., House, J.K. 2018. Short communication: Shedding of *Mycoplasma bovis* and antibody responses in cows recently diagnosed with clinical infection. *J Dairy Sci 101*, 584-589.

Jasper, D.E., 1981. Bovine mycoplasmal mastitis. *Adv Vet Sci Comp Med 25*, 121-157.

Jordan, A.G., Sadler, R.J., Sawford, K., van Andel, M., Ward, M., Cowled, B.D. 2021. *Mycoplasma bovis* outbreak in New Zealand cattle: An assessment of transmission trends using surveillance data. *Transbound Emerg Dis 0*, 1-15.

Maunsell, F., Brown, M.B., Powe, J., Ivey, J., Woolard, M., Love, W., Simecka, J.W. 2012. Oral inoculation of young dairy calves with *Mycoplasma bovis* results in colonization of tonsils, development of otitis media and local immunity. *PLoS One 7*, e44523.

Maunsell, F.P., Donovan, G.A. 2009. *Mycoplasma bovis* infections in young calves. *Vet Clin North Am Food Anim Pract 25*, 139-177.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle. *J Vet Intern Med 25*, 772-783.

Ministry of Primary Industries, the Office of the Chief Science Adviser. 2019. Report on appropriateness of classification of properties in the *Mycoplasma bovis* eradication response programme. https://www.mpi.govt.nz/dmsdocument/33696/direct, last accessed 1 April, 2021)

Ministry for Primary Industries, 2021. *Mycoplasma bovis* situation report. Latest updates on *Mycoplasma bovis* and infected properties.(https://www.mpi.govt.nz/biosecurity/mycoplasma-bovis/situation-report/, last accessed 1 April, 2021)

Murai, K., Lehenbauer, T.W., Champagne, J.D., Glenn, K., Aly, S.S. 2014. Cost-effectiveness of diagnostic strategies using quantitative real-time PCR and bacterial culture to identify contagious mastitis cases in large dairy herds. *Prev Vet Med 113*, 522-535.

Nielsen, P.K., Petersen, M.B., Nielsen, L.R., Halasa, T., Toft, N. 2015. Latent class analysis of bulk

tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma bovis*. *Prev Vet Med 121,* 338-342.

Pardon, B., Buczinski, S. 2020. Bovine respiratory disease diagnosis: what progress has been made in infectious diagnosis? *Vet Clin North Am Food Anim Pract 36*, 425-444.

Pardon, B., Callens, J., Maris, J., Allais, L., Van Praet, W., Deprez, P., Ribbens, S. 2020. Pathogen-specific risk factors in acute outbreaks of respiratory disease in calves. *J Dairy Sci 103,* 2556-2566

Parker, A. M., House, J.K., Hazelton, M.S., Bosward, K.L., Mohler, V.L., Maunsell, F.P., Sheehy, P.A. 2016. Milk acidification to control the growth of *Mycoplasma bovis* and *Salmonella Dublin* in contaminated milk. *J Dairy Sci 99*, 9875-9884.

Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House, J.K. 2018. A review of mycoplasma diagnostics in cattle. *J Vet Intern Med 32*, 1241-1252.

Parker, Alysia M., Shukla, A., House, J.K., Hazelton, M.S., Bosward, K.L., Kokotovic, B., Sheehy, P.A. 2016. Genetic characterization of Australian *Mycoplasma bovis* isolates through whole genome sequencing analysis. *Vet Microbiol 196*, 118-125.

Perez-Casal, J. 2020. Pathogenesis and virulence of *Mycoplasma bovis*. *Vet Clin North Am Food Anim Pract 36*, 269-278.

Pereyre, S., Tardy, F., Renaudin, H., Cauvin, E., Del Prá Netto Machado, L., Tricot, A., Benoit, F., Treilles, M., Bébéar, C. 2013. Identification and subtyping of clinically relevant human and ruminant mycoplasmas by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol 51*, 3314-3323.

Punyapornwithaya, V., Fox, L.K., Hancock, D.D., Gay, J.M., Richard Alldredge, J. 2012. Time to clearance of Mycoplasma mastitis: The effect of management factors including milking time hygiene and preferential culling. *Can Vet J 53*, 1119-1122.

Punyapornwithaya , V., Fox, L.K., Hancock, D.D., Gay, J.M., Wenz, J.R., Alldredge, J.R. 2011. Incidence and transmission of *Mycoplasma bovis* mastitis in Holstein dairy cows in a hospital pen: A case study. *Prev Vet Med 98*, 74-8.

Randall, L.P., Lemma, F., Koylass, M., Rogers, J., Ayling, R.D., Worth, D., Klita, M., Steventon, A., Line, K., Wragg, P., Muchowski, J., Kostrzewa, M., Whatmore, A.M. 2015. Evaluation of MALDI-ToF as a method for the identification of bacteria in the veterinary diagnostic laboratory. *Res Vet Sci 101*, 42-49.

Razin, S., Hayflick, L. 2010. Highlights of mycoplasma research - An historical perspective. *Biologicals 38*, 183-190.

Spergser, J., Macher, K., Kargl, M., Lysnyansky, I., Rosengarten, R. 2013. Emergence, re-emergence, spread and host species crossing of *Mycoplasma bovis* in the Austrian Alps caused by a single endemic strain. *Vet Microbiol 164*, 299-306.

Stats, 2019. Agricultural production statistics: June 2019 (final).

https://www.stats.govt.nz/information-releases/agricultural-production-statistics-june-2019-final

Timonen, A.A.E., Autio, T., Pohjanvirta, T., Häkkinen, L., Katholm, J., Petersen, A., Mõtus, K., Kalmus, P. 2020. Dynamics of the within-herd prevalence of *Mycoplasma bovis* intramammary infection in endemically infected dairy herds. *Vet Microbiol 242*, 108608.

Vähänikkilä, N., Pohjanvirta, T., Haapala, V., Simojoki, H., Soveri, T., Browning, G.F., Pelkonen, S., Wawegama, N.K., Autio, T. 2019. Characterisation of the course of *Mycoplasma bovis* infection in naturally infected dairy herds. *Vet Microbiol 231*, 107-115.

Van Driessche, L., Valgaeren, B.R., Gille, L., Boyen, F., Ducatelle, R., Haesebrouck, F., Deprez, P., Pardon, B. 2017. A deep nasopharyngeal swab versus nonendoscopic bronchoalveolar lavage for isolation of bacterial pathogens from preweaned calves with respiratory disease. *J Vet Intern Med 31*, 946-953.

Vyletělová, M. 2010. The survival of *Mycoplasma bovis* at different temperatures. *Czech J Food Sci 28,* 74-78.

Wawegama, N.K., Markham, P.F., Kanci, A., Schibrowski, M., Oswin, S., Barnes, T.S., Firestone, S.M., Mahony, T.J., Browning, G.F. 2016. Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for detection of *Mycoplasma bovis* infection in feedlot cattle. *J Clin Microbiol 54*, 1269-1275

SUMMARY

Mycoplasma bovis is a leading, primary cause of pneumonia, arthritis, otitis and mastitis in cattle, resulting in impaired animal welfare and huge economic losses in all cattle sectors worldwide. This small bacterium lost its cell wall and several physiological mechanisms through evolution, whereupon it acquired inherent resistance against many conventional antimicrobials (*e.g.* penicillines, cephalosporines, sulfonamides, ..). Next to this natural resistance, *M. bovis* may acquire resistance against other antimicrobials as well. Currently, isolation and identification of *M. bovis* by culture takes 1-2 weeks, and subsequent antimicrobial susceptibility testing is currently not performed in routine diagnostics. No standard protocol is available and the lack of clinical breakpoints limits the translation of *in vitro* results to clinical outcome predictions of antimicrobial treatment. At a higher price, faster identification is possible with PCR (2 days). Although diagnostic accuracy of PCR is expected to be higher than culture, scientific information on this matter is limited.
To be able to control *M. bovis* and start appropriate antimicrobial treatment immediately, there is a great need for rapid and reliable diagnostic tools for this pathogen. However, next to control, prevention of *M. bovis* spreading into/within the herd is also very important. How *M. bovis* is exactly transmitted, and whether there are specific *M. bovis* strains associated with antimicrobial resistance or sectors, has not been elucidated yet. Key factors for successful control and prevention are the formulation of specific biosecurity protocols and guidelines targeted to *M. bovis*. To achieve this, a rapid diagnosis of infected or carrier animals and better insights into the spread of *M. bovis* between herds, sectors, and countries are needed.

Therefore, the general aim of this thesis was to develop new Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) and nanopore sequencing based diagnostic methods for rapid identification, strain typing, and antimicrobial susceptibility testing of *M. bovis*, and to apply those methods on Belgian field samples, gaining better insight into the epidemiology of *M. bovis*.

In the general introduction (Chapter 1) a literature overview is provided, presenting current state-of-the-art on disease course, risk factors and treatment of *M. bovis*. Subsequently, the many different diagnostic techniques available for identification, strain typing, and susceptibility testing are described. Next to the existing techniques, more innovative techniques, such as MALDI-TOF MS and nanopore sequencing and their potential as rapid diagnostic methods are explained.

In the first experimental study different methods were explored to identify *M. bovis* cultures grown on solid medium with MALDI-TOF MS (Chapter 3.1). The most straight-forward method, being the direct transfer method, is broadly applied for most bacteria, but faced several problems for *M. bovis* identification. In this study, these problems were better identified and it was shown that medium-related peaks (mostly obtained from horse serum and colistin) can result in false positive *Mycoplasma alkalescens* and *Mycoplasma arginini* identification. Unfortunately, it was not possible to obtain a more reliable direct transfer protocol. Therefore, in Chapter 3.2 the identification of *M. bovis* with MALDI-TOF MS from liquid medium was further explored and optimized. Here it was shown that identification was possible within 24 hours after inoculation of one colony from a solid medium into liquid medium. Supplementing pleuropneumonia-like organism broth (PPLO-broth) with pyruvate prolonged the possibility of *M. bovis* identification to at least 120 hours after inoculation. Also, supplementation with antimicrobials prevented overgrowth with other bacteria, and did not influence the identification score.

Although with the previous two methods, a step towards more rapid identification of *M. bovis* was set, prior isolation of *M. bovis* from any sample is still necessary and could easily take 5-10 days. Therefore, methods to identify *M. bovis* directly from bronchoalveolar lavage fluid (BALf) with MALDI-TOF MS (Chapter 3.3) and nanopore sequencing (Chapter 3.4) were developed and validated in a Bayesian latent class model on 104 and 100 BALf from calves, respectively. It was possible to identify *M. bovis* with MALDI-TOF MS within 2-3 days with a sensitivity and specificity of 86.6% (CI95%: 69.4-97.6%) and 86.4% (76.1-93.8%), respectively. While sensitivity and specificity of nanopore sequencing were 77.4% (58.6-92.3%) and 97.3% (91.1-99.7%), respectively. Also when 5 BALf were pooled, both methods were still reliable, and therefore very cost-effective possibilities. In addition, the in Belgium widely used selective-indicative agar method based on lipase-activity, which was never validated before using a large number of field samples, showed a sensitivity of 70.5% (52.1-87.1%) and specificity of 93.9% (85.9-98.4). All three methods are useful in routine laboratories, depending on the diagnostic needs of the applicant.

Currently the prevalence of *M. bovis* in the Belgian dairy and beef sector is estimated at 30%, whereas 100% of the veal calf herds tested positive. Together with the high antimicrobial use in the veal sector, the question has been raised whether there is a possible reservoir of multi-resistant and sector-specific *M. bovis* strains in this sector, as previously shown for other

respiratory bacteria. To better understand the molecular epidemiology and genetic relatedness of different *M. bovis* isolates, the full genome of 100 Belgian *M. bovis* isolates collected from dairy, beef and veal herds was obtained using nanopore sequencing (Chapter 4). A single nucleotide polymorphism (SNP) analysis was performed and the phylogenetic tree showed five separate genomic clusters of *M. bovis* isolates and one outlier circulating in Belgium between 2014 and 2019. No sector-specific isolates and no association with spatial location in Belgium were identified. At world-scale, the Belgian *M. bovis* isolates clustered together with European, American and Israeli strains. These results contribute to emphasizing the importance of purchase protocols and biosecurity to prevent *M. bovis* from entering the country or herd.

In Chapter 5.1, antimicrobial susceptibility testing of 141 *M. bovis* isolates retrieved from Belgian dairy, beef and veal calf herds was performed with broth microdilution. Minimum inhibitory concentration values were used to establish the epidemiological cut-off (ECOFF) with visual and statistical methods to distinguish the population in wild type *M. bovis* and those with acquired antimicrobial resistance (non-wild type). The results showed high percentages of acquired resistance for macrolides (tilmicosin, tylosin, and gamithromycin), but no acquired resistance for tetracyclines (oxytetracycline, doxycycline). Only little acquired resistance was observed for florfenicol, gentamicin, and tiamulin, while there was limited acquired resistance to enrofloxacin. Only *M. bovis* isolates from beef cattle or the third genomic cluster had a significantly higher change to have acquired resistance against gamithromycin than those collected from other sectors or genomic clusters. These results support the current national formulary for respiratory disease associated with *M. bovis*, recommending florfenicol as first choice, and oxytetracycline and macrolides as second choice. Possibly, a small remark for gamithromycin is needed, as higher risk for acquired resistance for this antimicrobial was seen in beef cattle. *In vitro* susceptibility testing results should be interpreted carefully, as the association with *in vivo* efficacy has not confirmed yet, due to the lack of clinical breakpoints.

Finally, in Chapter 5.2, upgraded genomes derived from Chapter 4 and the susceptibility data from Chapter 5.1 were combined to compare genotype and phenotype antimicrobial susceptibility of *M. bovis* isolates. A genome wide association study to reveal genetic markers for antimicrobial resistance in *M. bovis* and verifying the ECOFF values obtained by the previously used different methods was executed. Many point mutations were associated with

antimicrobial resistance against the critically important antibiotics of the macrolide (A2058G in the 23S rRNA gene, Gln83His in the L22 protein) and fluoroquinolone classes. For enrofloxacin the combination of different mutations in the *GyrA* and *ParC* gene showed the step-wise acquired resistance. Also previously described mutations for tilmicosin (G478A mutation in 23S rRNA alleles), and new markers for gentamicin (A1408G and G1488A in 16S rRNA) were identified. The visual estimation of de ECOFF showed to be a reliable method, although statistical methods can help when step-wise resistance results in difficult to interpret "tailing". Even when phenotypical resistance is not yet obtained, in case of first-step mutations it should be discouraged to use fluoroquinolones as antimicrobial therapy, as selection pressure will eventually result in phenotypical resistance as well.

In the general discussion (Chapter 6), the innovations in *M. bovis* diagnostics achieved with this thesis are discussed. In the second part, practical recommendations for diagnostics in *M. bovis* outbreak management, purchase policy, and eradication or herd status certificates are proposed.

In this thesis new methods to identify, strain type, and access the antimicrobial susceptibility of *M. bovis* were developed. When rapid identification of *M. bovis* with MALDI-TOF MS (Chapter 3.3) is followed by the determination of antimicrobial resistance with nanopore sequencing (Chapter 5.2) it is now possible to obtain identification, strain typing and an antibiogram for critically important antibiotics within 3-5 days. This is a major step towards better control of *M. bovis* in clinical outbreaks and prevent herd infection when purchasing animals. Together with these new methods, also substantial epidemiological information came to light, showing the importance of a more national approach for the prevention of introducing *M. bovis* into the herd and country.

SAMENVATTING

Mycoplasma bovis is een primaire oorzaak van pneumonie, artritis, otitis en mastitis bij runderen. Deze ziekte resulteert in verminderd dierenwelzijn en leidt wereldwijd tot grote economische verliezen. Door evolutie is deze kleine bacterie zijn celwand en verschillende fysiologische mechanismes verloren. Hierdoor werd het resistent tegen vele conventionele antibiotica (bijv. penicillines, cefalosporinen en sulfonamiden). Naast deze natuurlijke resistentie, heeft *M. bovis* ook resistentie verworven tegen andere antibiotica.
Momenteel duurt het isoleren en identificeren van *M. bovis* met cultuur 1 à 2 weken en antimicrobiële resistentiebepalingen worden niet routinematig gedaan. Er is namelijk geen standaard protocol beschikbaar. Daarnaast zijn er ook geen klinische breekpunten beschikbaar, waardoor het voorspellen van de klinische uitkomst aan de hand van *in vitro* resultaten niet mogelijk is. Tegen een hogere prijs is snellere identificatie mogelijk met PCR (2 dagen). Echter, ondanks dat men verwacht dat de diagnostische accuraatheid van PCR hoger is dan deze van cultuur, is er maar weinig wetenschappelijke informatie over beschikbaar.
Om *M. bovis* onder controle te krijgen en direct te starten met de juiste antimicrobiële therapie, is het van groot belang een snelle en betrouwbare diagnostische test te hebben. Naast de controle is het ook van groot belang zowel de introductie van *M. bovis* in de kudde, als de spreiding binnen de kudde tegen te gaan. Hoe *M. bovis* precies wordt overgedragen en of er specifieke *M. bovis* stammen bestaan die geassocieerd zijn met antimicrobiële resistentie of bepaalde sectoren is nog niet duidelijk. Sleutelfactoren voor het succes van controle en preventie zijn het formuleren van specifieke bioveiligheidsprotocollen en richtlijnen voor *M. bovis*. Hiervoor is snelle diagnostiek van geïnfecteerde dieren of dragers en een beter inzicht in de spreiding van *M. bovis* in kuddes, sectoren en landen nodig.

Daarom was het algemene doel van deze thesis om snelle diagnostische methoden te ontwikkelen met behulp van MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) en nanopore sequencing voor identificatie, stamtypering en antimicrobiële gevoeligheidstesten voor *M. bovis* en deze toe te passen op Belgische veldstalen voor een beter inzicht in de epidemiologie van *M. bovis*.

In de algemene introductie (Hoofdstuk 1) is een overzicht gegeven van de literatuur over de meest recente inzichten in ziekteverloop, risicofactoren en de behandeling van *M. bovis*. Vervolgens worden de verschillende diagnostische technieken beschikbaar voor identificatie, stamtypering en antimicrobiële gevoeligheidstesten beschreven. Naast de bestaande

methoden, worden ook de meer innovatieve methoden, zoals MALDI-TOF MS en nanopore sequencing en hun potentieel als snelle diagnostische methoden uitgelegd.

In de eerste experimentele studie werden verschillende methoden onderzocht om *M. bovis* van agarcultuur te kunnen identificeren met MALDI-TOF MS (Hoofdstuk 3.1). De meest voor de hand liggende methode, de 'direct transfer method', wordt grootschalig toegepast voor de meeste bacteriën, maar kan problematisch zijn voor *M. bovis*-identificatie. In deze studie werden de problemen beter in kaart gebracht en het werd aangetoond dat mediumgerelateerde pieken (vooral afkomstig van paardenserum en colistine) resulteerde in vals-positieve *Mycoplasma alkalescens* en *Mycoplasma arginini* identificatie. Helaas was het niet mogelijk om een betrouwbaarder 'direct transfer protocol' te realiseren. Daarom werd in Hoofdstuk 3.2 de identificatie van *M. bovis* met MALDI-TOF MS vanuit vloeibaar medium verder onderzocht en geoptimaliseerd. Hier werd aangetoond dat identificatie mogelijk is 24 uur na inoculeren van één kolonie afkomstig van agar in vloeibaar medium. Supplementeren van pleuropneumonia-like organism broth (PPLO-broth) met pyruvaat verlengde de mogelijkheid om *M. bovis* te identificeren tot minstens 120 uur na inoculatie. Daarnaast bleek dat de supplementatie met antibiotica de contaminatie met andere bacteriën voorkwam en de identificatiescore niet beïnvloedde.

Hoewel er in de twee voorgaande studies stappen in de richting van een snellere identificatie van *M. bovis* werden gezet, is isolatie vanuit een staal, wat 5 tot 10 dagen duurt, nog altijd nodig. Daarom werden er methoden voor identificatie van *M. bovis* rechtstreeks uit bronchoalveolaire lavages (BAL) ontwikkeld met MALDI-TOF MS (Hoofdstuk 3.3) en nanopore sequencing (Hoofdstuk 3.4). Deze methoden werden gevalideerd op respectievelijk 100 en 104 BAL-stalen met een Bayesiaanse analyse. Het was mogelijk om *M. bovis* met een sensitiviteit van 86.6% (CI95%: 69.4-97.6%) en specificiteit van 86.4% (76.1-93.8%) binnen 2 tot 3 dagen te identificeren met MALDI-TOF MS, terwijl de sensitiviteit en specificiteit van nanopore sequencing respectievelijk 77.4% (58.6-92.3%) en 97.3% (91.1-99.7%) waren. Ook wanneer 5 BAL-stalen werden gepoold, bleken deze methoden betrouwbaar en dus zeer kostenefficiënt. Daarnaast werd een nog niet eerder gevalideerde, maar veel in België toegepaste, selectief-indicatieve agarmethode gebaseerd op lipase-activiteit, onderzocht. Deze methode toonde een sensitiviteit van 70.5% (52.1-87.1%) en specificiteit van 93.9% (85.9-98.4).

Momenteel is de prevalentie van *M. bovis* in de melkvee- en vleesveesector geschat op 30%, terwijl in de vleeskalversector 100% van de bedrijven positief test. Samen met het hoge antibioticagebruik in de vleeskalversector werd de vraag gesteld of deze sector een mogelijk reservoir is voor multiresistente of sectorspecifieke *M. bovis*-stammen, zoals aangetoond werd voor andere respiratoire bacteriën. Om de moleculaire epidemiologie en het genetisch verwantschap van verschillende *M. bovis*-isolaten beter te begrijpen, werd het volledige genoom van 100 Belgische *M. bovis* isolaten afkomstig van melkvee, vleesvee en vleeskalveren verkregen met nanopore sequencing (Hoofdstuk 4). Een single nucleotide polymorfisme (SNP) analyse werd uitgevoerd en de fylogenetische boom toonde dat vijf aparte genetische clusters van *M. bovis*-isolaten en een uitschieter circuleerden in België tussen 2014 en 2019. Er werden geen sectorspecifieke isolaten of een associatie met de locatie in België geïdentificeerd. Op wereldschaal werd wel gezien dat de Belgische *M. bovis*-isolaten samen clusterden met Europese, Amerikaanse en Israëlische isolaten. De resultaten van dit onderzoek onderstrepen het belang van aankoopprotocollen en bioveiligheid om het binnenkomen van *M. bovis* op een bedrijf en in het land te voorkomen.

In Hoofdstuk 5.1 werden er antimicrobiële gevoeligheidsbepalingen uitgevoerd door middel van broth microdilutie op 141 *M. bovis*-isolaten afkomstig van Belgische melkvee-, vleesvee- en vleeskalverbedrijven. Met behulp van visuele en statistische methoden werd de epidemiologische cut-off (ECOFF) waarde bepaald op basis van de minimum inhibitoire concentraties van de isolaten. De ECOFF verdeelt de *M. bovis* populatie in 'wild type' (zonder verworven resistentie) en 'niet-wild type' (met verworven antimicrobiële resistentie). De resultaten toonden een hoog percentage verworven resistentie voor macroliden (tilmicosine, tylosine en gamithromycine), maar geen verworven resistentie tegen tetracyclines (oxytetracycline, doxycycline). Er werd minimale resistentie gezien tegen florfenicol, gentamicine en tiamuline, terwijl er beperkte resistentie werd gezien voor enrofloxacine. Alleen *M. bovis*-isolaten afkomstig van vleesvee of het derde genetische cluster hadden een significant hogere kans op verworven resistentie tegen gamithromycine dan deze afkomstig van andere sectoren of genetische clusters. De resultaten steunen de huidige nationale formularia voor respiratoire ziekten geassocieerd met *M. bovis*, waar florfenicol als eerste keuze en oxytetracycline en macroliden als tweede keuze worden aangeraden. Een kleine kanttekening voor gamithromycine kan worden gemaakt, gezien de hogere kans op verworven resistentie van *M. bovis* tegen dit antibioticum bij vleesvee. Resultaten van *in vitro* gevoeligheidstesten moeten echter wel voorzichtig worden

geïnterpreteerd omdat de associatie met *in vivo* efficaciteit niet is bevestigd, gezien het gebrek aan klinische breekpunten.

Ten slotte werden in Hoofdstuk 5.2 de geüpgradede genomen van Hoofdstuk 4 en de gevoeligheidstesten uit Hoofdstuk 5.1 gecombineerd om het antimicrobiële resistentie-fenotype met het genotype te associëren. Een 'genome wide association study' om genetische markers voor antimicrobiële resistentie in *M. bovis* te identificeren en eerder gebruikte ECOFF waarden te evalueren, werd uitgevoerd. Verschillende puntmutaties werden geassocieerd met antimicrobiële resistentie tegen kritisch belangrijke antibiotica van de klassen der macroliden (A2058G in het 23S rRNA gen, Gln83His in het L22 eiwit) en fluoroquinolonen. Verschillende combinaties van mutaties in de genen *GyrA* en *ParC* toonden de stapsgewijze resistentieverwerving voor enrofloxacine. Ook eerder beschreven mutaties voor tilmicosine (G478A mutaties in de 23S rRNA allelen) en nieuwe markers voor gentamicine (A1408G en G1488A in het 16S rRNA) werden geïdentificeerd. De visuele schatting van de ECOFF waarde bleek een betrouwbare methode, al kunnen statistische methoden bijdragen bij moeilijk te interpreteren resultaten, zoals bij stapsgewijze resistentie ('tailing'). Zelfs wanneer fenotypische resistentie nog niet is bereikt, zou in gevallen van eerste-stapmutaties het gebruik van fluoroquinolonen als antimicrobiële therapie moeten worden afgeraden, omdat de selectiedruk uiteindelijk toch zal resulteren in fenotypische resistentie.

In de algemene discussie (Hoofdstuk 6) worden eerst de innovaties in *M. bovis*-diagnostiek besproken die behaald zijn in deze thesis. Vervolgens worden praktische aanbevelingen voorgesteld omtrent diagnostiek van *M. bovis* in uitbraakmanagement, aankoopprotocollen, eradicatie en potentiële certificaten op kuddeniveau.

In deze thesis werden nieuwe methoden ontwikkeld voor de identificatie en stamtypering van *M. bovis*, alsook voor het verkrijgen van inzicht in zijn antimicrobiële gevoeligheid. Wanneer snelle identificatie van *M. bovis* met MALDI-TOF MS (Hoofdstuk 3.3) wordt opgevolgd door antimicrobiële resistentiebepaling via nanopore sequencing (Hoofdstuk 5.2), is het nu mogelijk om identificatie, stamtypering en een antibiogram van kritisch belangrijke antibiotica te realiseren binnen 3 tot 5 dagen. Dit is een grote stap richting betere controle van *M. bovis* in klinische uitbraken en preventie van infecties in de kudde ten gevolge van aankoop. Samen met deze nieuwe methoden is ook substantiële epidemiologische informatie aan het licht gekomen die het belang van een meer nationale aanpak voor de introductiepreventie van *M. bovis* in kuddes en het land aantoont.

CURRICULUM VITAE

Jade Bokma was born on 14 August 1991 in Tegelen, the Netherlands. After finishing high school at the Alfa College in Groningen, and one year of Psychobiology at the University of Amsterdam, she started studying Veterinary Medicine at the University of Antwerp in 2011. After her bachelor's degree she went on to study at Ghent University, where she obtained her master's degree in Veterinary Medicine, option Ruminants, graduating *summa cum laude* in 2017. Her master's thesis ("Risk factors for antimicrobial use in veal calves") was awarded as the best master thesis on antimicrobial resistance by AMCRA (Antimicrobial Consumption and Resistance in Animals).

Immediately after graduation, Jade Bokma started her PhD research at the Department of Large Animal Internal Medicine (Faculty of Veterinary Medicine, Ghent University) financed by the Belgian Federal Public Service, Health, Food Chain Safety and Environment (FOD, RF 17/6313, MALDIRESP/MA), which was supervised by Prof. dr. B. Pardon, Dr. F. Boyen, Prof. dr. P. Deprez and Prof. dr. F. Haesebrouck. This research project focused on rapid antimicrobial susceptibility testing using MALDI-TOF MS and nanopore sequencing in bacterial pathogens from lung and udder in cattle. In addition, she was involved in a Veepeiler project (Animal Health Care Flanders) concerning *Mycoplasma bovis* in Flanders. She participated in the day and night shifts as veterinarian in the clinic for ruminants, took care of the analysis of clinical samples, and contributed to the education of master students. She also obtained the certificate of the Doctoral Training Programme of Life Sciences and Medicine.

Jade Bokma is (co-)author of several papers in (inter)national peer-reviewed journals and professional literature. She is also reviewer for international journals, and gave multiple presentations at (inter)national conferences.

Jade Bokma werd geboren op 14 augustus 1991 te Tegelen, Nederland. Na het behalen van haar middelbareschooldiploma aan het Alfa College in Groningen en een jaar Psychobiologie aan de Universiteit van Amsterdam, startte ze in 2011 met de studie Diergeneeskunde aan de Universiteit Antwerpen. Na de bachelor vervolgde zij haar studie Diergeneeskunde aan de Universiteit Gent waar ze in 2017 het diploma van dierenarts (optie herkauwers) met de grootste onderscheiding ontving. Haar masterthesis “Risicofactoren voor antibioticumgebruik bij witvleeskalveren” werd beloond met de prijs voor beste masterthesis over antibioticaresistentie, uitgereikt door het AMCRA (Antimicrobial Consumption and Resistance in Animals).

Onmiddellijk na afstuderen trad ze in dienst van de vakgroep Inwendige Ziekten van de Grote Huisdieren (Faculteit Diergeneeskunde, Universiteit Gent) als doctoraatsstudent op een project gefinancierd door het FOD Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu. Onder begeleiding van Prof. dr. B. Pardon, Dr. F. Boyen, Prof. Dr. P. Deprez en Prof. dr. F. Haesebrouck focuste zij zich op snelle antimicrobiële gevoeligheidsbepalingen met behulp van MALDI-TOF MS en nanopore sequencing van bacteriële pathogenen afkomstig van de long en uier van runderen (RF 17/6313, MALDIRESP/MA). Daarnaast was zij betrokken bij het Veepeiler project (Dierengezondheidszorg Vlaanderen) omtrent *Mycoplasma bovis* in Vlaanderen, nam zij deel aan de dag-, nacht- en weekenddiensten als dierenarts in de kliniek voor herkauwers, zorgde zij voor de analyse van klinische stalen en droeg zij bij aan het onderwijs van de masterstudenten diergeneeskunde. Daarnaast behaalde zij haar certificaat van het Doctoral Training Programme of Life Sciences and Medicine.

Jade Bokma is (mede)auteur van meerdere publicaties in (inter)nationale wetenschappelijke tijdschriften. Ze is ook reviewer van internationale wetenschappelijke tijdschriften en was meermaals spreker op (inter)nationale congressen.

BIBLIOGRAPHY

PUBLICATIONS

Bokma J., Dewulf J., Deprez P., Pardon B. 2018. Risk factors for antimicrobial use in food-producing animals: disease prevention and socio-economic factors as the main drivers? *Vlaams Diergeneeskundig Tijdschrift 87*, 188-200.

Van Driessche L., **Bokma J.**, Gille L., Ceyssens PJ, Sparbier K., Haesebrouck F., Deprez P., Boyen F., Pardon B. 2018. Rapid detection of tetracycline resistance in bovine *Pasteurella multocida* isolates by MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA). *Scientific Reports 8.*

Bokma J., Van Driessche L., Gille L., Haesebrouck F., Deprez P., Boyen F., Pardon B. 2019. Optimizing identification of *Mycoplasma bovis* by MALDI-TOF MS. *Research in Veterinary Science 125*, 185-188.

Bokma J., Boone R., Deprez P., Pardon B. 2019. Risk factors for antimicrobial use in veal calves and the association with mortality. *Journal of Dairy Science 102*, 607-618.

Van Driessche L., **Bokma J.**, Deprez P., Haesebrouck F., Boyen F., Pardon B. 2019. Rapid identification of respiratory bacterial pathogens from bronchoalveolar lavage fluid in cattle by MALDI-TOF MS. *Scientific Reports* 9.

Bokma J., Boone R., Deprez P., Pardon B. 2020. Short communication: Herd-level analysis of antimicrobial use and mortality in veal calves: do herds with low usage face higher mortality? *Journal of Dairy Science 103*, 909-914.

Bokma J., Pardon B., Deprez P., Haesebrouck F., Boyen F. 2020. Non-specific, agar medium-related peaks can result in false positive *Mycoplasma alkalescens* and *Mycoplasma arginini* identification by MALDI-TOF MS. *Research in Veterinary Science 130*, 139-143.

Bokma J., Van Driessche L., Deprez P., Haesebrouck F., Vahl M., Weesendorp E., Deurenberg R.H., Pardon B., Boyen F. 2020. Rapid identification of *Mycoplasma bovis* strains from bovine bronchoalveolar lavage fluid with MALDI-TOF MS after enrichment procedure. *Journal of Clinical Microbiology 58*, e0004-20.

Vereecke N., **Bokma J.**, Haesebrouck F., Nauwynck H., Boyen F., Pardon B., Theuns S. 2020. High quality genome assemblies of *Mycoplasma bovis* using a taxon-specific Bonito basecaller for MinION and Flongle long-read nanopore sequencing. *BMC Bioinformatics 21*, 517.

Bokma J., Vereecke N., De Bleecker K., Callens J., Ribbens Sl., Nauwynck H., Haesebrouck F., Theuns S., Boyen F., Pardon B. 2020. Phylogenomic analysis of *Mycoplasma bovis* from Belgian veal, dairy and beef herds. *Veterinary research 51*, 121.

Bokma J., Gille L., De Bleecker K., Callens J., Haesebrouck F., Pardon B., Boyen F. 2020. Antimicrobial susceptiblity of *Mycoplasma bovis* isolates from veal, dairy and beef herds. *Antibiotics 9*.

Pas M.L., Vanneste K., **Bokma J.**, Van Driessche L., De Keersmaecker S.C.J., Roosens N.H., Haesebrouck F., Boyen F., Pardon B. 2020. Case report: multidrug resistant *Raoultella ornithinolytica* in a septicemic calf. *Frontiers in Veterinary Science 26*.

Bokma J., Vereecke N., Nauwynck H., Haesebrouck F., Theuns S., Boyen F., Pardon B. Genome Wide Association Study reveals genetic markers for antimicrobial resistance in *Mycoplasma bovis. Submitted.*

Bokma J., Vereecke N., Pas M.L., Chantillon L., Haesebrouck F., Vahl M., Deurenberg R.H., Weesendorp E., Theuns S., Boyen F., Pardon B. 2021. Diagnostic accuracy of nanopore sequencing in the identification of *Mycoplasma* species from bovine bronchoalveolar lavage fluid. *Submitted.*

Jourquin S., **Bokma J.**, De Cremer L., van Leenen K., Vereecke N., Pardon B. 2021. Cure rate and required therapy length of florfenicol and oxytetracycline in an outbreak of *Mycoplasma bovis* pneumonia using ultrasonographic lung reaeration as a cure criterion. *Submitted*.

ORAL PRESENTATIONS

Bokma J., Boone R., Deprez P., Pardon B. 2018. Antimicrobial use and risk factors in veal calves. *Vet2030 research symposium, Faculty of Veterinary Medicine, Ghent University*, Ghent, Belgium.

Van Driessche L., De Cremer L., van Leenen K., **Bokma J.**, Gille L., Deprez P., Boyen F., Pardon B. 2018. Multiresistant *Gallibacterium anatis* in a clinical outbreak of infectious bronchopneumonia in beef cattle. *The 30th World Buiatrics Congress*, Sapporo, Japan.

Bokma J., Boone R., Deprez P., Pardon B. 2018. The relationship between antimicrobial use and herd level mortality as a welfare indicator in veal calves. *The 9th International conference on Antimicrobial Agents in Veterinary Medicine,* Rome, Italy.

Van Driessche L., **Bokma J.**, Gille L., Ceyssens P.J., Sparbier K., Haesebrouck F., Deprez P., Boyen F., Pardon B. 2018. Rapid tetracycline resistance detection in bovine *Pasteurella multocida* isolates by MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA). *The 7th Bruker MALDI Biotyper, BeLux Users meeting*. Brussels, Belgium.

Van Driessche L., **Bokma J.**, Gille L., Ceyssens P.J., Sparbier K., Haesebrouck F., Deprez P., Boyen F., Pardon B. 2018. Rapid detection of tetracycline resistance in bovine *Pasteurella multocida* isolates by MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA). *The 9th International conference on Antimicrobial Agents in Veterinary Medicine,* Rome, Italy.

Bokma J., Van Driessche L., Deprez P., Hasebrouck F., Pardon B., Boyen F. 2019. Direct identification of *Mycoplasma bovis* from bronchoalveolar lavage fluid with MALDI-TOF MS. *The European Mycoplasma Conference*, London, United Kingdom.

Bokma J., Van Driessche L., Deprez P., Boyen F., Pardon B. 2019. Direct MALDI-TOF MS identification of bovine pathogens retrieved from non-endoscopic broncho-alveolar lavages. *The 8th Bruker Microbiology and Diagnostics BeLux Users Meeting*, Brussels, Belgium.

Bokma J., Van Driessche L., Deprez P., Haesebrouck F., Boyen F., Pardon B. 2019. Identification of Mycoplasma bovis directly from bronchoalveolar lavage fluid with MALDI-TOF MS. *The European Bovine Congress 2019: Your veterinary toolbox 2025*, ‘s Hertogenbosch, The Netherlands.

De Cremer, L., van Leenen, K., Van Driessche, L., **Bokma, J**., Deprez, P., Pardon, B. 2019. Lung ultrasonography to reduce antimicrobial therapy length in an outbreak of *Mycoplasma bovis*. *The European Bovine Congress 2019: Your veterinary toolbox 2025*, 's Hertogenbosch, The Netherlands.

Bokma, J., Vereecke, Gille, L., Van Driessche, L., De Bleecker, K., Nauwynck, H., Haesebrouck, F., Theuns, S., Boyen, F., Pardon B. Antimicrobial susceptiblity testing and strain typing on *Mycoplasma bovis* from beef, dairy and veal farms. *The 23th Congress of the International Organization for Mycoplasmology (IOM)*, Tel aviv, Israel. (Submitted – postponed due to COVID-19)

Bokma, J., Vereecke, Gille, L., Van Driessche, L., De Bleecker, K., Nauwynck, H., Haesebrouck, F., Theuns, S., Boyen, F., Pardon B. Genomics-based epidemiology and antimicrobial susceptibility of *Mycoplasma bovis* isolates from veal, dairy, and beef herds. *The 31th World Buiatrics Congress*, Madrid, Spain. (Submitted – postponed due to COVID-19)

Bokma J. Opportunities in rapid diagnosis of respiratory pathogens: what is the current situation and what can we expect? 2020. *European Veterinary Conference Voorjaarsdagen*, The Hague, The Netherlands. (invited - postponed due to COVID-19))

Bokma J. *Mycoplasma bovis*: a mini-review about a maxi pathogen. 2020. *European Veterinary Conference Voorjaarsdagen*, The Hague, The Netherlands. (invited - postponed due to COVID-19)

Bokma J., Vereecke N., De Bleecker K., Callens J., Nauwynck H., Haesebrouck F., Theuns S., Pardon B., Boyen F. 2020. Phylogenomic analysis and antimicrobial susceptibility data of *Mycoplasma bovis* from different Belgian cattle production systems. *2nd International Conference of the European College of Veterinary Microbiology*. Online.

Vereecke N., **Bokma J.**, Haesebrouck F., Nauwynck H., Theuns S., Pardon S., Boyen F. 2021. *Mycoplasma bovis* point mutations as genetic markers for antimicrobial resistance revealed by genome wide association study. *Nanopore London Calling*, 2021. Online.

POSTER PRESENTATIONS

Bokma J., Boone R., Deprez P., Pardon B. 2018. Trends in antimicrobial use in veal calves (2014-2016) and associated risk factors. *Quantification, Benchmarking and Stewardship of Veterinary Antimicrobial Usage, First International Conference*", Ghent, Belgium.

Bokma J., Boone R., Deprez P., Pardon B. 2018. Does reduction of antimicrobial use increase mortality in veal calves? *Veterinary Epidemiology and Economics (VEE), annual meeting*, Brussels, Belgium.

Van Driessche L., De Cremer L., van Leenen K., **Bokma J.**, Gille L., Deprez P., Boyen F., Pardon B. 2018. Multiresistant *Mannheimia haemolytica* isolates as a cause of therapy failure for bronchopneumonia in beef farms. *The 9th International conference on Antimicrobial Agents in Veterinary Medicine*". Rome, Italy.

Bokma J., Pardon B., Van Driessche L., Gille L., Deprez P., Haesebrouck F., Boyen F. 2018. Optimizing identification of *Mycoplasma bovis* by MALDI-TOF MS. *The 9th International conference on Antimicrobial Agents in Veterinary Medicine*. Rome, Italy.

Bokma J., Van Driessche L., Deprez P., Hasebrouck F., Pardon B., Boyen F. 2019. Non-specific, medium-related peaks can result in false positive *Mycoplasma* spp. identification with MALDI-TOF MS. *The European Mycoplasma Conference*, London, United Kingdom.

Bokma J., van Leenen K., De Cremer, L., Boyen F., Pardon B. 2019. *Mycoplasma bovis* peritonitis and omental bursitis after C-section in two Belgian Blue cows. *The European Bovine Congress 2019: Your veterinary toolbox 2025*, 's Hertogenbosch, The Netherlands.

Pas M., **Bokma J.**, Van Driessche L., Vanneste K., De Keersmaecker S., Roosens N., Boyen F., Pardon B. 2019. Neonatal sepsis caused by *Raoultella ornithinolytica* in a calf. *The European Bovine Congress 2019: Your veterinary toolbox 2025*, 's Hertogenbosch, The Netherlands.

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België, ik zie u graag

Ik woon niet waar ik ben geboren
Ergens tussen Antwerpen en Gent
In een land waar ik na al die jaren
Soms nog steeds een vreemde ben
Land van lang vervlogen tijden
Land van leven achter luiken
Land van mensen die iets zeggen
Met de kunst van het ontwijken

Ik zie u graag, ik zie u graag
Met al je chaos en je schoonheid
Al je bier en troosteloosheid
Ik zie u nog altijd graag

Land van ingehouden woede
Land van uitgesproken stilte
Land van lelijkheid die mooi is
Land van talen die verschillen
Land van kroegen, kathedralen
Land van weergaloze vrouwen
Land van grijze dorpen, zotte morgen
Ik zal altijd van u houden

Ik zie u graag, ik zie u graag
Land van chaos en schoonheid
Bier en achterbaksheid
Ik zie u nog altijd graag

Dit liefdeslied is ongenaakbaar
Want ik hou van jou zoals je bent
Met al je voors en al je tegens
Niemand heeft jou ooit getemd
Land dat kan omarmen
Land dat kan verstoten
Land om te verlaten
Land om terug te komen
Land waar ik altijd een vreemde zal blijven
Land dat ik nooit echt zal begrijpen
maar ik zie u graag
Ik zie u nog altijd graag

Stef Bos